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The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 57 JANUARY, 1961 NO. 1

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Letter From the Editors ---------- 1

The Whip With the Silver Handle. John H. Dingle, Sc.D., M.D., Cleveland, Ohio --- 3

Clinical and Experimental

Turnover of Plasma Orosomucoid in Man. Sherman Weisman, M.D., Barry Goldsmith, M.D., Richard Winzler, Ph.D., and Mark H. Lepper, M.D., Chicago, Ill. ------ 7

Different Rheumatoid Factors in the Serum of One Patient With Rheumatoid Arthritis. Ralph Heimer, Ph.D., Edith R. Schwartz, A.M., and Richard H. Freyberg, M.D., New York, N. Y. ---------- 16

Red Cell Electrolytes in Patients With Edema. Edward Kessler, M.D., M. Robert Levy, M.D., and Royal L. Allen, Jr., A.B., East Orange, N. J. ---------- 32

A Micro Modification of a Method for the Measurement of Globulin in Cerebrospinal Fluid and Its Application in Diagnosis of Multiple Sclerosis. Albert Hanok, M.S., New York, N. Y. ---------- 42

Effect of Estrogen Administration on Hydrocortisone Metabolism in Man. Leon J. Marks, M.D., Gilbert R. Friedman, M.D., and Frederick J. Duncan, M.D., Boston, Mass. ---------- 47

A Method for Demonstrating Probable Synthesis of Protein by Bone Marrow Cells. Wayne E. Truax, M.D., Juanita Bray, B.A., and John E. Perry, M.D., Galveston, Texas ---------- 54

Studies on Erythropoiesis. XVI. Response to a Single Dose of Erythropoietin in Polycythemic Mouse. Edward Filmanowicz, B.S., and Clifford W. Gurney, M.D., Chicago, Ill. ---------- 65

Phenylalanine Tolerance Tests in Patients With Leukemia. Hwa L. Wang, Ph.D., and Harry A. Waisman, M.D., Ph.D., Madison, Wis. ---------- 73

Ornithine Carbamyl Transferase Activity in Human Serum in Diseases of the Liver and the Biliary System. Hans Reichard, M.D., Stockholm, Sweden ---------- 78

(Contents continued on inside front cover)

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Contents—*(Continued from front cover)*

Effect on Isolated Rat Diaphragm of Ammonium and Related Nitrogen Containing Compounds. Robert E. Bolinger, M.D., Kansas City, Kan. With the Technical Assistance of B. J. Slinker ---------- 88

Plasma Unesterified Fatty Acid Concentrations in Neoplastic Disease. Peter S. Mueller, M.D., and Donald M. Watkin, M.D., Bethesda, Md. ---------- 95

Measurement of Residual Urine With I^{131}-Labeled Diodrast. P. J. Mulrow, M.D., A. Huvos, M.D., and D. L. Buchanan, M.D., New Haven, Conn. ---------- 109

Pulmonary Vascular Changes After Intravenous Injection of Thromboplastin in Rabbits. Scott R. Inkley, M.D., Louis Gillespie, Jr., M.D. and Simon Koletsky, M.D., Cleveland, Ohio ---------- 114

Urinary Excretion of 3-Methoxy-4-Hydroxymandelic Acid After Several Stress Situations. M. Lawrence Berman, Ph.D., and Julia A. Pettitt, M.T. (A.S.C.P.), Wright-Patterson Air Force Base, Ohio ---------- 126

Laboratory Methods

Use of Precursor Product Relationships in Determining Serum Albumin Half-Life. Sherman Weissman, M.D., Donald P. Tschudy, M.D., Habeeb Bacchus, M.D., and Marjoria Eubanks, B.S., Bethesda, Md. ---------- 136

Prothrombin Measurement by Two Stage Technique With Optimum Molarity of Calcium. O. Herman Dreskin, M.D., Cincinnati, Ohio ---------- 147

Aortic Bypass Procedure for Study of Gastric Microcirculation. Horacio A. Dolcini, M.D., Isidoro Zaidman, M.D., and Seymour J. Gray, M.D., Ph.D., Boston, Mass. 152

Rapid Assay Procedures for Tritium-Labeled Water in Body Fluids. B. E. Vaughan, Ph.D., and E. A. Boling, M.D., San Francisco, Calif. ---------- 159

A Simple Isotope Method for Recording the Achilles Tendon Reflex in Myxedema. Alton R. Sharpe, Jr., Lieutenant (MC) USNR, Postsmouth, Va. With the Technical Assistance of A. V. McKeon, H. T. Dent, M. V. Donahue, and H. J. Van Wagner, Jr. ---------- 165

(Editorial and Business Communications on page 26)

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The Journal of Laboratory and Clinical Medicine

VOL. 57 JANUARY, 1961 No. 1

LETTER FROM THE EDITORS

As of January 1, Dr. William H. Daughaday, Associate Professor of Medicine at the Washington University School of Medicine, became Editor of the JOURNAL OF LABORATORY AND CLINICAL MEDICINE. He has chosen Dr. Neal S. Bricker, Assistant Professor of Medicine, as his Associate Editor. Dr. William Sodeman and Dr. Donald Seldin have completed, respectively, 6 year and 3 year terms on the Board. On behalf of the Central Society for Clinical Research and the Editorial Board, we wish to express our appreciation for their valuable services.

Dr. Robert Hodges, Dr. Charles Owen, and Dr. Marvin Siperstein have accepted appointments and Dr. Robert Good has accepted reappointment on the Board of Editors.

During the past 6 years the Editors and Board members have called upon many of their associates and colleagues in other universities to aid them in the evaluation of manuscripts. Those who have participated in this capacity in the past year are:

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We wish to take this opportunity to thank the past and present members of the Editorial Board for their excellent services to the JOURNAL and to the Central Society for Clinical Research. We would also like to express our appreciation for the cooperation received from The C. V. Mosby Company.

We have considered it an honor and a privilege to serve the Central Society for Clinical Research and The C. V. Mosby Company as Editors of this JOURNAL and we extend to the new Editors, Dr. Daughaday and Dr. Bricker, our congratulations and best wishes.

WILLIAM D. ROBINSON, M.D., EDITOR
WILLIAM H. BEIERWALTES, M.D., ASSOCIATE EDITOR

THE WHIP WITH THE SILVER HANDLE

JOHN H. DINGLE, SC.D., M.D.

CLEVELAND, OHIO

ONE hundred and eighty years ago Benjamin Franklin, one of our country's sagest men, said:

> We may perhaps learn to deprive large masses of their Gravity, and give them absolute Levity, for the sake of easy Transport. Agriculture may diminish its Labour and double its Produce; all Diseases may by sure means be prevented or cured, not excepting even that of Old Age, and our Lives lengthened at pleasure even beyond the antediluvian Standard. O that moral Science were in as fair a way of Improvement. . . .[1]

Franklin's statement was, in part, remarkably prophetic. Much that he postulated has now come to pass. We have learned how to deprive large masses of their gravity and have given them absolute levity, as indicated by the satellites now in orbit. The labor of agriculture has been decreased at least threefold and its productivity increased four- or fivefold. We can specifically cure as well as prevent many diseases, but by no means all. Our life expectancy has increased approximately 50 per cent in the last 50 years. But the prophecy has not been completely fulfilled, for while the problems of old age have become increasingly evident, in reality we as yet know little about coping with them. And, beyond even Franklin's prophetic vision, we are faced with new hazards, such as radiation and air pollution, that man is creating for himself. Dealing with such problems as these requires changes in medical practice and medical research. This morning I should like to consider with you one approach—based on concepts of clinical medicine, preventive medicine, epidemiology, and biostatistics—that I believe has much to offer in developing solutions to the present and future problems of disease and of maintenance of health.

These concepts are concerned with the broad aspects of disease and health: with the widest possible scope of the factors responsible for the initiation, distribution, and progression of the phenomena of disease and health. In the case of disease, they require consideration of such factors as the causative agents, be they specific or multiple, including predisposing, precipitating and perpetuating factors, the range of susceptibility, the clinical manifestations as they occur in all degrees of severity from mild to fatal, the factors in spread, and the effect of environment. When adequate data have been obtained regarding such aspects, and have been integrated and thoughtfully analyzed,

[1] Presidential address, Twenty-third Annual Meeting of the Central Society for Clinical Research, Chicago, Illinois, Nov. 4 and 5, 1960.

it should then be possible to arrive at effective means of prevention and of treatment. Thus, although these concepts are not directly concerned with public health programs or therapeutic regimes, they are concerned with obtaining the basic knowledge on which such programs may be developed. Moreover, it may not be necessary to have all of this information for the solution or partial solution of some problems. Dysentery, typhoid, cholera, and other enteric diseases were dramatically controlled in the western world by the introduction of environmental sanitary procedures based on epidemiologic studies long before the etiologic agents and other aspects of the diseases were known. Other problems may well be similarly amenable to control.

What, then, are the components necessary to this approach and how are the concepts put into practice? Obviously the disease, or problem, must be studied in a population of human beings. The first requirement, to my mind at least, is the clinical approach—a sound knowledge of clinical medicine, the ability to diagnose or to recognize and identify a disease in an individual person, for if the problem is not recognized, there is no problem. The second is the epidemiologic and biostatistical approach, which requires the study of a defined population in which the disease or problem occurs. The latter approach seeks to answer such questions as: Where does the disease occur? When? Who acquires it and who does not? Is its occurrence related to age, sex, or race? How is it transmitted? Pertinent information regarding the problem may come from many sources—the diagnostic laboratory, the basic science laboratories, and the disciplines ancillary to medicine. When the data from all these sources are welded together, they will offer a description of the behavior of the disease which should provide a basis for prevention and control of the problem.

These concepts are not new. They have been held by good doctors for years, although they have been more widely accepted and extended in the last two decades. They are the basis for what Alan Gregg termed "mature medicine."

It is important, however, to consider who are the "good doctors" who practice mature medicine. What type of person is best qualified to utilize this approach? To my mind he is the well-trained physician who is interested in patients and is competent in various aspects of clinical investigation. He should be skilled in the art of observation. He should be able to accept and assimilate data from his own and other disciplines. He should be open-minded yet critical, and he should be sufficiently mature so that he no longer thinks of his patients as a "cardiac," an "asthmatic," a "Billroth II," a "liver," or a "spleen." He can then begin to think of his patient as a person, of his family, of the social milieu in which the disease is occurring, and of the behavior of the disease in the population.

Thus far, we have been concerned chiefly with disease. Now what about the other side of the coin—the matter of health? Health is hard to define. Most attempts have defined it negatively as the "absence of disease." Dissatisfied with such a statement, the World Health Organization defined it positively

as a "state of complete physical, mental and social well-being and not merely an absence of disease or infirmity." This is an admirable statement, but what does it mean? Is a man unhealthy who has a pimple on his nose, dislikes his boss, and has an argument with his spouse before breakfast? He may be energetic, creative, imaginative, tolerant, and productive in all other respects, which would suggest that he was healthy. If you, the members of this audience, were asked to list the symptoms of disease, none of you would have any difficulty in filling a page. But if you were asked to list the symptoms of health, I doubt if many of you could list ten. There are, of course, other definitions of health. For example, Osler is reputed to have defined the state of health as one in which the head is cool and the feet are warm. Francis[2] considered health to be adequate structure and function with reserves adequate to absorb those stresses which may reduce reserves to the extent that tolerance is exceeded and disorder results. But it is apparent that we have no specific and adequate definition of health as we do of disease and we cannot study health effectively until we can define it. Much more work is necessary in order to describe health and to develop criteria and methods for measuring it.

Now what are the problems that can be approached in terms of the concepts I have presented? The answer is: almost any of the problems that are based on the observation of man. They may arise from a consideration of the leading causes of death or disability, which I need not list for this audience. They may come from the elegant and basic work that is being done in many laboratories in the fields of microbiology, genetics, biochemistry, metabolism and so forth. They are now arising as relatively new problems, such as new diseases resulting from mutations in causative organisms or in man himself, the emergence of bacteria formerly considered harmless, as causes, for example, of pneumonia and pyelonephritis, the effects of new food stuffs and food preservatives, radiation hazards, air pollution, and many others.

I wish to urge here that some clinical investigators maintain a broad approach to the many difficult problems we face in medicine today and tomorrow in an attempt to define more adequately the natural history of disease and the biology of man. I believe that this approach can be used most effectively by the clinically trained investigator who is working with people. Please do not misunderstand me. I am not raising the argument of the descriptive versus the experimental approach or the clinical versus the basic science approach. Every possible approach should be used in accordance with the interest and training of the investigators themselves. Leads to the solution of difficult problems, such as the phenomena of aging, may come from anywhere. But the recognition and description of these phenomena may best be done by physicians. Who else is in a better position ultimately to answer such questions as: What factors lead to the development of degenerative diseases? Can their influence be minimized or even reversed? Do the minor illnesses of infancy and childhood have any relation to the rapidity of aging or to illness and disability in old age? I am sure that you could add many more.

And finally, any physician, if willing, can utilize this approach and can use the data he obtains from his clinical associations with patients. You all remember the "Gold-Headed Cane" that was carried by the leading London practitioners in the seventeenth and eighteenth centuries as a symbol of their knowledge, authority, success, and elegance. These men had prestige, but they were artists, not scientists, although they were patrons of science. But as Dana Atchley[3] pointed out: "The art of medicine [at that time] was so devoid of scientific knowledge that pomp and ceremony were highly useful therapeutic adjuncts."

There was also a symbol for the outstanding country practitioner who drove a horse and buggy from house to house to care for his patients, and that was a "Whip with the Silver Handle." Perhaps one of the finest of these doctors was Edward Jenner who observed and realized the significance of cowpox on the fingers of milkers in relation to resistance to smallpox and who developed the first specific immunizing procedure in man—vaccination. Although its symbolism has changed somewhat, the "Gold-Headed Cane" may still be found in many of our clinics and laboratories. I hope that some of our clinical investigators will continue to be those who figuratively carry the "Whip with the Silver Handle."

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CLINICAL AND EXPERIMENTAL

TURNOVER OF PLASMA OROSOMUCOID IN MAN

SHERMAN WEISMAN, M.D.,* BARRY GOLDSMITH, M.D., RICHARD WINZLER, PH.D., AND MARK H. LEPPER, M.D.
CHICAGO, ILL.

The half-life of I^{131}-iodinated orosomucoid was determined in patients convalescing from several acute diseases, patients with active parenchymal liver disease, and patients with subacute inflammatory disease. Two lots of orosomucoid prepared by $(NH_4)_2SO_4$ precipitation from Fraction VI of human plasma were used. After iodination the bulk of the radioactivity migrated with orosomucoid in paper electrophoresis at pHs 4.5 and 8.6. Radioactivity in the patients' sera after administration of the labeled compound also migrated. The concentration of orosomucoid in the plasma showed a more rapid and variable dropoff initially than I^{131}-albumin which was used as a control which may have reflected instability of the compound after iodination. However, after 5 to 7 days the concentration curves followed a straight line which allowed calculation of the half-life. This was slightly over 5 days in all three groups of patients. The rate at which isotope disappeared from the plasma and extravascular space could be accounted for by dialyzable isotope excreted in the urine. There was no evidence that unchanged protein was sequestered in the body. Since there were major differences in the serum orosomucoid concentration in these patients it was inferred that there was an increased rate of synthesis in patients with inflammatory disease and a decrease in those with parenchymal liver disease which led to high and low serum concentrations, respectively.

THE seromucoid fraction of human plasma has been defined as that group of macromolecular substances not precipitable by boiling in slightly acid pH,[1, 2] but soluble in 0.6N perchloric acid and not soluble in 1M phosphotungstic acid.[3] Recent studies have demonstrated the chemical and immunologic heterogeneity of this fraction.[4-8] Biologic heterogeneity is also strongly implied by the fact that shifting ratios of carbohydrate to protein and glucosamine to galactosamine have been found in this fraction in various disease states.[9, 10] It is well established that there is a net elevation of the concentration of this group of substances in the plasma of patients with malignant neoplasms, acute and chronic infectious diseases, and various collagen diseases, whereas there is a tendency for the concentration to be low in patients with nephrosis and parenchymal liver disease.[11-13]

Orosomucoid is a relatively well-defined major glycoprotein component of seromucoid. It is an acidic substance with a molecular weight of 41,000 that contains 15 per cent hexose as galactose and mannose, 14 per cent glucosamine,

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a small amount of fucose, and 16 per cent sialic acid.[4, 14] The remainder of the molecule is made up of amino acids. The sialic acid is believed to be in a terminal position in the molecule and responsible for its unusually low isoelectric point (2.7). Although its elevation in disease parallels the increase in the total seromucoid fraction,[15] there is an almost complete lack of information on its biologic functions in the body, its sources and mode of formation, and its distribution, degradation, and metabolism. The results of studies after administration of orosomucoid labeled with radioactive iodine are reported in this article.

METHODS

Two separate lots, A and B, of orosomucoid were prepared from Fraction VI separated from pooled normal human plasma by the method of Weimer and collaborators.[16] This involved precipitation of the bulk of the proteins by half saturated ammonium sulfate, at pH 3.7, followed by precipitation of orosomucoid with saturated ammonium sulfate at pH 3.7. The protein was stored as the lyophilized powder until iodinated. Both preparations showed single negatively charged bands on paper electrophoresis at pHs 8.6 and 4.5 with less than 5 per cent of the protein lying outside of these bands. At pH 8.6 the mobility corresponded to alpha$_1$ protein and at pH 4.5, to M-$_1$ protein.[17]

Iodination was performed commercially* using the following procedure. Seventy to 100 mg. of the orosomucoid was taken up in water and the pH was adjusted to about 8.0 with a minimal amount of sodium bicarbonate. One half milligram of potassium iodide and 2 to 3 mc. of carrier-free sodium iodide I^{131} was added. The solution was cooled to about 0° C. and the iodine was freed by the controlled addition of 0.001N hypochlorous acid. Unreacted iodide was separated by passage of the mixture through an ion exchange resin, Dowex 2X8. At no point was the temperature above 26° C. A total of three separate iodinations of orosomucoid were performed, producing lots A1, B1, and B2 of iodinated orosomucoid.

The final material contained more than 99 per cent of its radioactivity in a nondialyzable form. Paper electrophoresis followed by radioautography was performed on a mixture of radioiodinated orosomucoid with a large excess of unlabeled orosomucoid. Some serum samples from patients who were receiving radioactive orosomucoid were also subjected to paper electrophoresis. Paper electrophoresis was done on a Spinco electrophoresis apparatus by standard methods. The buffers used were pH 8.6, 0.1M barbital and pH 4.5, 0.1M acetate. Radioautography was accomplished by pinning the strips of dried paper from the electrophoresis to an x-ray film and exposing the film in a cassette for periods varying from one hour to two weeks according to the radioactivity of the sample. The paper strips were stained with bromphenol blue to visualize the protein. The results obtained with the labeled orosomucoid (Fig. 1) showed considerable heterogeneity of the labeled sample. However, most of the radioactivity had the mobility of an alpha$_1$ globulin at pH 8.6, and at pH 4.5 showed a broad negatively charged band. Thus it may be concluded that part of the orosomucoid was altered during the iodination. Samples of serum taken 5 to 14 days after administration of labeled orosomucoid, however, showed radioactivity only in the albumin-α_2 and M$_1$ areas suggesting that the altered orosomucoid is rapidly cleared. This is in accord with serum activity studies reported in Fig. 2, and would lend credence to the data obtained after 5 to 7 days.

Total plasma seromucoid was estimated by the method of Winzler.[5] Radioactivity of serum samples was determined by counting 3 ml. specimens in a plastic tube, using a well-type sodium iodide crystal scintillation counter with four pi geometry. Each sample was counted for at least 3 minutes and ten thousand counts above background. Samples were

*The radioiodination of orosomucoid was carried out by Abbott Laboratories. Radioactive albumin was also obtained from this company.

counted only if they had at least 80 c.p.m. above background. A standard made by dilution of the material injected into the patients was included on each counting day. The activity of each sample was corrected to day 0 by multiplying the observed activity by the ratio of activity of the standard on day 0 to its activity on the counting day.

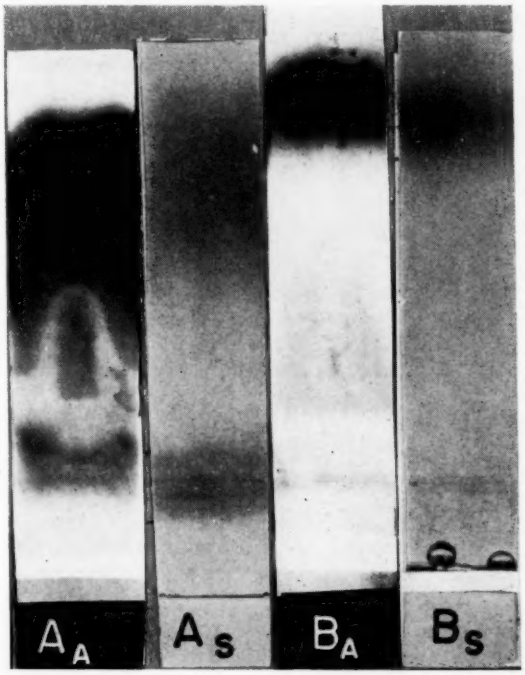

Fig. 1.—Paper electrophoretograms of solution of I^{131}-labeled orosomucoid with a large excess of unlabeled orosomucoid of the same lot. The cathode is at the top of the figure. Strips *A* were run at a pH of 4.5, A_S was stained for protein, and A_A is its radioautograph. Strips *B* were run at a pH of 8.6 B_S was stained for protein and B_A is the radioautograph.

For each study 1 ml. of normal saline containing 2 mg. of orosomucoid labeled with about 50 μc of I^{131} was injected from a calibrated syringe into the tubing through which a saline solution was being given intravenously. All patients were given 10 drops of a saturated solution of potassium iodide twice daily during the study period, beginning 1 to 3 days before the isotope was administered.

Blood specimens were drawn into heparinized syringes, centrifuged, and the supernatant plasma was used for counting. Tissue samples were counted by placing a weighed amount of fresh tissue, generally 1 to 2 grams, in a plastic tube, tamping it down, and adding enough water to make a 3 ml. volume. Three milliliter aliquots of 24 hour urine specimens were placed in a plastic tube and counted directly.

Labeled orosomucoid was administered to fourteen subjects. Blood samples were obtained at approximately 15 minutes, 2 hours, one half day, and one day after the injection was given, then daily for a week and every second day for the next one and one half to two weeks. Determinations were continued at least 16 days in most subjects.

Urinary excretion curves were made from measurements of the total isotope excreted in at least twelve 24 hour urine collections obtained during the first two and one half weeks after the injection. In cases where the total recovery of isotope was computed, daily 24 hour urine collections were obtained for the first 2 weeks.

Seromucoid levels were determined on all patients at least twice during the study and all exhibited relatively constant levels, suggesting but not proving that the orosomucoid levels were also stable.

Five patients, including two who had been studied with orosomucoid, were injected with 50 μc of commercially prepared radioiodinated serum albumin and followed in the same manner as the patients given orosomucoid.

The subjects given the labeled glycoprotein fell roughly in three categories. Seven patients were considered normal since they only had chronic neurologic disability or were in the latter phases of recovery from an episode of myocardial infarction or bacterial endocarditis. There was no fever and no evidence of active infection, malignancy, collagen disease, or endocrine disorder, and little or no heart failure. A second group was made up of four patients with parenchymal liver disease without the complicating factors listed except for a sterile buttock abscess in patient E. W. All of this group of patients except R. W.[2] had a low serum albumin with a reversed albumin to globulin ratio. The third group was made up of three patients with an active infection or a collagen disease who had a fever at least of a low grade throughout most of the study. An additional patient with cor pulmonale died of a pulmonary embolus on the sixth day of the study.

RESULTS AND DISCUSSION

Each of five plasma samples taken at various intervals within the first week after the patients had received the orosomucoid contained between 50 and 60 per cent of the radioactivity which was precipitable by 0.6M perchloric acid. This is a somewhat greater proportion than the 30 to 50 per cent of the seromucoid fraction coprecipitated with plasma proteins previously noted,[5, 10] and may be related to the alteration of part of the orosomucoid previously mentioned. The perchloric acid-soluble radioactivity was quantitatively precipitated by phosphotungstic acid. Fifteen plasma samples chosen from several patients

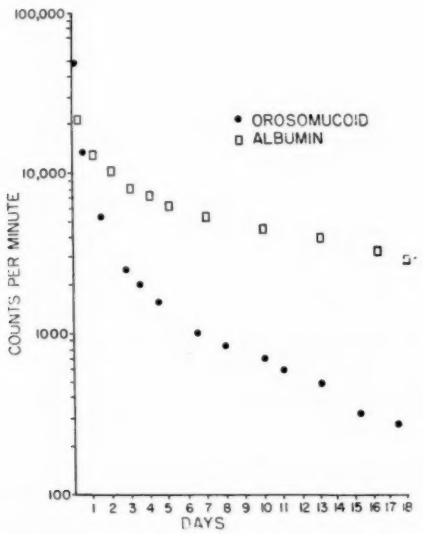

Fig. 2.—Comparison of results obtained after the administration of I^{131}-labeled orosomucoid with those obtained after administration of I^{131}-labeled albumin. The logarithms of the counts per minute found in the plasma on the indicated day after administration of the labeled compound are plotted.

TABLE I. HALF-LIFE AND EXCRETION OF RADIOACTIVITY AFTER INJECTION OF I^{131}-TAGGED ALBUMIN

PATIENT	DISEASE	PLASMA T½ (DAYS)	DAILY URINE EXCRETION CURVE T½ (DAYS)	% ISOTOPE ACCOUNTED FOR IN URINE
J. D.	Decerebrate 2 degrees to intracranial hemorrhage	15	—	—
E. W.	Laennec's cirrhosis with fever and buttock abscess	16	16	53
O. C.	Laennec's cirrhosis	18	17	55
C. O.	Laennec's cirrhosis	12	10	65
C. L.	Myocardial infarct	13	13.5	56

TABLE II. HALF-LIFE AND EXCRETION OF RADIOACTIVITY AFTER INJECTION OF I^{131}-TAGGED OROSOMUCOID

CATEGORY	PATIENT	LOT OF I^{131} OROSOMUCOID	PLASMA T½ (DAYS)	DAILY URINE EXCRETION CURVE T½ (DAYS)	% ISOTOPE ACCOUNTED FOR IN THE URINE	PLASMA SEROMUCOID MEAN LEVEL (MG. PROTEIN/100 ML.)
Normal	P. B.	A1	5.5	—	—	91
	A. J.	A1	4.5	4.5	79	96
	A. K.	B1	7	4.5	102	103
	E. J.	B1	5	4	105	110
	N. K.	B2	5.5	5	88	188
	M. P.	B2	5.5	4.5	—	54
	R. W.	B1	5.75	3.75	81	111
Liver disease	O. C.	A1	6.25	5.75	—	76
	R. W.[2]	A1	5.75	4.75	—	81
	O. B.	B1	6	5	92	46
	E. W.	B1	7.5	4	93	87
Inflammation	R. V.	A1	6.25	—	—	154
	A. C.	B2	4.75	—	—	192
	L. N.	B2	4.50	3.5	77	138

at random within the first 2 weeks of the study all showed that the radioactivity migrated throughout the albumin and fast alpha$_1$ region at pH 8.6. At pH 4.5 none of the radioactivity migrated toward the cathode and most migrated as did M_1 or orosomucoid in all samples examined. Some radioactivity was also found in a band corresponding to M_2.[17] These results are consistent with the assumption that most of the radioactivity was associated with orosomucoid which was not altered to such a degree as to change its electrophoretic mobility during the course of its survival in the blood stream.

Fig. 2 shows typical curves obtained when the logarithm of the plasma radioactivity was plotted against time after administration of labeled albumin and orosomucoid. Curves obtained with iodinated albumin were entirely consistent with those displayed in the relevant literature.[18] The amount of radioactivity in the plasma after administration of orosomucoid showed a more rapid and variable initial dropoff than that obtained with albumin. This again suggests alteration of some of the orosomucoid during iodination. After 5 to 7 days, however, both the orosomucoid and albumin plasma radioactivity curves

TABLE III. TISSUE RADIOACTIVITY IN PATIENTS WHO RECEIVED I^{131}-TAGGED OROSOMUCOID

PATIENT	DISEASE	DAY OF STUDY ON WHICH TISSUE WAS OBTAINED	PLASMA RADIOACTIVE DAY OF TISSUE STUDY	TISSUE RADIOACTIVITY (C.P.M./GM. WET WEIGHT)							
				THYROID	LIVER	LUNG	MUSCLE	SKIN	KIDNEY	SPLEEN	PUS
R. V.	Bacterial endocarditis	10	332	—	108	234	130	86	202	158	—
A. M.	Cor pulmonale	6	168	280	73	101	66	—	109	60	—
E. W.	Laennec's cirrhosis and gluteal abscess	6	183	—	—	—	—	—	—	—	130

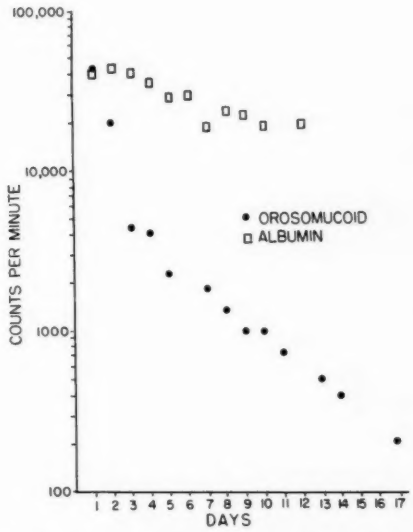

Fig. 3.—Comparison of results obtained after the administration of I^{131}-labeled orosomucoid with those obtained after administration of I^{131}-labeled albumin. The logarithms of the counts found in the urine are plotted for the indicated days.

in almost all cases were very well approximated by a straight line. Because of the rapid initial loss of radioactivity, pool sizes and dilution volumes could not be calculated accurately and are not presented.

The graph of urine daily radioactivity excretion curves were much less smooth than the plasma curves, as may be seen by comparing Figs. 2 and 3. The urine curves clearly reflect the more rapid early removal of isotope from orosomucoid, and the relative absence of this phenomenon with albumin. After 5 to 7 days the urine curves also could be roughly fitted by a straight line. Attempts to estimate the rate of degradation of orosomucoid from the slope of the graph obtained by plotting the total isotope injected minus cumulative isotope excretion versus time[18] were particularly inaccurate for this substance. After the fifth day as much as 75 per cent of the isotope injected had been excreted, so that even a 5 per cent error in the estimation of the amount of isotope injected would produce a 20 per cent error in the estimate of the amount remaining in the body. Furthermore, a small error in collection or counting of an early sample would produce a large distortion of the latter portion of the curve.

The data for cumulative isotope excreted in the urine during the first 2 weeks, expressed as per cent of injected isotope, are presented in Tables I and II for the patients for whom it was available. If, as indicated in the tables, the average half-life for albumin was 14 days and for orosomucoid was 5 to 6 days the isotope collected in the urine would account in a quantitative fashion for the disappearance of the protein from the plasma and the extravascular space. These data, therefore, do not suggest that trapping of the glycoprotein in any compartment other than plasma and extravascular fluid is a major factor in the metabolism of this substance.

If constant orosomucoid levels were assumed in the subjects studied, the degradation rate may be taken as indicative of the fractional replacement rate of plasma orosomucoid, ultimately by synthetic processes. The half-times of the linear portions of the plasma curves averaged 5.2, 5.4, and 5.2 for the three groups of patients studied with orosomucoid. Thus the absolute production rate in the group with liver disease has been somewhat lower, and in the group with inflammations higher than in the normal subjects because of the lower and higher plasma levels, respectively. However the rate of orosomucoid synthesis as so measured was not a sensitive indicator of the patients' status.

A few stool specimens were tested and all showed less than 10 per cent of the radioactivity shown by the urine specimen of the same day. Fecal loss of radioactivity was thus negligible for the purpose of the present study.

Dialysis of urine samples showed that more than 95 per cent of the radioactivity passed through the membrane.

Tissue specimens were obtained on two patients who died during the course of the study (Table III). Both had been in shock at least 12 hours before death. The activity of the thyroid of patient A. M. demonstrated the relative efficiency of the potassium iodide in preventing thyroidal accumulation of isotope. There was no definite evidence for disproportionate localization of isotope in any other tissue. Pus was aspirated from a sterile gluteal abscess in a third patient and showed no striking concentration of isotope.

The relatively rapid rate of iodinated orosomucoid metabolism observed here is consistent with the rapid appearance and disappearance of isotope in similar plasma fractions, observed when C^{14}-labeled glucose was administered to guinea pigs[19] or to rabbits,[20] and with the rapid turnover of alpha$_1$ globulins in the rat, observed with S^{35}-tagged protein.[21]

SUMMARY

Orosomucoid, iodinated with I^{131}, was administered to convalescent patients, patients with parenchymal liver disease, and patients with subacute inflammation. Although considerable heterogeneity of the labeled orosomucoid was apparent, the biologic half-life of orosomucoid could be determined after disappearance of the altered protein. The half-life in all three groups was slightly over 5 days. The rate at which isotope disappeared from plasma and extravascular space could be, within the limits of experimental error, accounted for as dialyzable isotope excreted in the urine. There was no evidence that unchanged protein was sequestered in the body. It was inferred that there was an increased rate of synthesis in patients with inflammatory disease and a decrease in those with parenchymal liver disease which led to high and low serum concentrations, respectively.

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DIFFERENT RHEUMATOID FACTORS IN THE SERUM OF ONE PATIENT WITH RHEUMATOID ARTHRITIS

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The occurrence of a number of unusual macroglobulins has been noted in the serum of a patient with rheumatoid arthritis. On examination in the analytic ultracentrifuge small amounts of macroglobulin, sedimenting either more rapidly or more slowly than the normal 19S macroglobulin were noted. A scheme for the isolation of rheumatoid factors was devised that resulted in the recovery of four distinct serum fractions. Each fraction was free of low molecular weight contaminants and possessed serologic activities characteristic of the rheumatoid factor. The first fraction, eluted after its adsorption onto the antigen-antibody complex, sheep erythrocyte stroma–rabbit antisheep serum, contained a main component sedimenting at 19.7S, which on electrophoresis migrated ahead of gamma globulin. The minor component sedimented at approximately 27S. The fraction was highly reactive with serologic reagents employing cells sensitized with rabbit antibody as well as with latex particles coated with human gamma globulin. Three additional macroglobular fractions were obtained from that portion of the serum which failed to adsorb onto the sheep erythrocyte stroma–rabbit antisheep serum complex. These fractions were obtained on step-wise elution of a euglobulin preparation applied onto DEAE cellulose. The fractions contained either a 19.5S, a 22.2S, or a 17.5S macroglobulin as the major component. The macroglobulins with such sedimentation coefficients were eventually isolated. The macroglobulins reacted with latex particles coated with human gamma globulin, but not with sheep cells sensitized with rabbit antibody. The heterogeneity of the rheumatoid factors seems to be a characteristic feature of sera of individuals with rheumatoid arthritis.

THE subject of rheumatoid factors has recently been extensively reviewed.[1, 2] The serum of individuals with rheumatoid arthritis contains rheumatoid factors. These are macroglobulins which agglutinate carrier cells sensitized with rabbit antibody or with human gamma globulin. Attempts to isolate them have been undertaken by various techniques such as alcohol fractionation,[3] column chromatography,[4-7] precipitation by human gamma globulin,[4, 8] adsorption onto human red cells coated with pneumococcal polysaccharide and sensitized with rabbit antipneumococcus serum,[9] onto human A cells sensitized with rabbit antihuman A serum,[9] and onto sheep cell stroma sensitized with rabbit antisheep serum.[10] The occurrence of more than one rheumatoid factor in a single

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serum sample was shown by Lospalluto and Ziff,[4] and work leading to similar conclusions was also reported by this laboratory.[7] Although precise information about the chemical characteristics of the rheumatoid factors is still lacking, preliminary studies on their amino acid composition[20] suggest the nonidentity and patient specificity of these macroglobulins. The possibility exists, however, that alteration of structure and therefore changes in serologic behavior could have been produced by cumbersome and often drastic purification procedures.

Accordingly, a study was undertaken to determine the individuality of the various purified macroglobulins obtained by fractionation of serum samples of one patient with rheumatoid arthritis.

MATERIALS

Serum was obtained from a 66-year-old, white woman with classic rheumatoid arthritis. She had a typical clinical course, definite x-ray changes, but no subcutaneous nodules. The patient has been treated with corticosteroids since 1950. Five separate pools of serum from various bleedings were used in the experiments. Samples B to D were stored in the freezer for periods up to 6 months; samples A and E were stored in the refrigerator and used within one week. Serum sample A was obtained 2 years prior to serum sample E.

METHODS

Electrophoresis.—Moving boundary electrophoresis was performed in a Perkin Elmer apparatus in diethylbarbiturate buffer at pH 8.6 ($\mu = 0.1$), and in phosphate buffer (Sorensen) at pH 7.0 ($\mu = 0.45$). Zone electrophoresis on starch was carried out according to the method of Kunkel[11] in diethylbarbiturate-saline buffer at pH 8.6 ($\mu = 0.1$) and in phosphate buffer at pH 7.6 ($\mu = 0.3$) at 4° C. using 8 to 10 volts per centimeter of starch. Paper electrophoresis was performed in a Spinco electrophoresis apparatus, model R, in a diethylbarbiturate buffer at pH 8.6 ($\mu = 0.1$).

Ultracentrifugation.—All ultracentrifuge analyses were carried out in a Spinco ultracentrifuge, model E, at a rotor temperature of 20° C. For the determination of the sedimentation coefficients of the isolated macroglobulins a cell with a double sector center piece at 47,660 r.p.m. was used. In analyses of serum samples or macroglobular concentrates of such sera a single sector cell at 59,780 r.p.m. was used. Sedimentation coefficients were corrected to standard conditions (water, 20° C.). Concentration dependent sedimentation constants were determined for the various isolated macroglobulins. Viscosimetric measurements of the solvent for the macroglobulins (0.15M phosphate buffer, pH 5.5) were carried out in an Ostwald type viscosimeter at 25° C.; the density of the solvent was determined in a Moore–Van Slyke pyknometer of 1.0 ml. volume at 20° C. as well as at 25° C.

Preparative ultracentrifugation was carried out in the Spinco ultracentrifuge, model E, with rotor K at 37,020 r.p.m. (147,090 g), using tubes of 36 ml. capacity or of smaller volume with appropriate adapters. Density gradient ultracentrifugation was performed in a sucrose-phosphate buffer (pH 5.5, 0.15M) solution, with a gradient varying from 50 to 5 per cent sucrose. The material to be fractionated was layered on top of the gradient solution in tubes of varying capacity and spun for 3 hours at 147,090 g. After centrifugation a tiny hole was cut in the bottom of the tube, as described by Edelman and coauthors,[19] for dropwise collection of fractions.

Fractionation of Serum.—Fractionation of the serum was carried out in two separate sets of experiments with serum sample C, comprising 109 ml. of serum, and with serum sample D, comprising 90 ml. of serum. Fig. 1 is a flow sheet of the purification procedure.

1. *Purification of macroglobulin I:* The procedure for purifying this component of the rheumatoid factors by use of sheep erythrocyte stroma sensitized with homologous rabbit

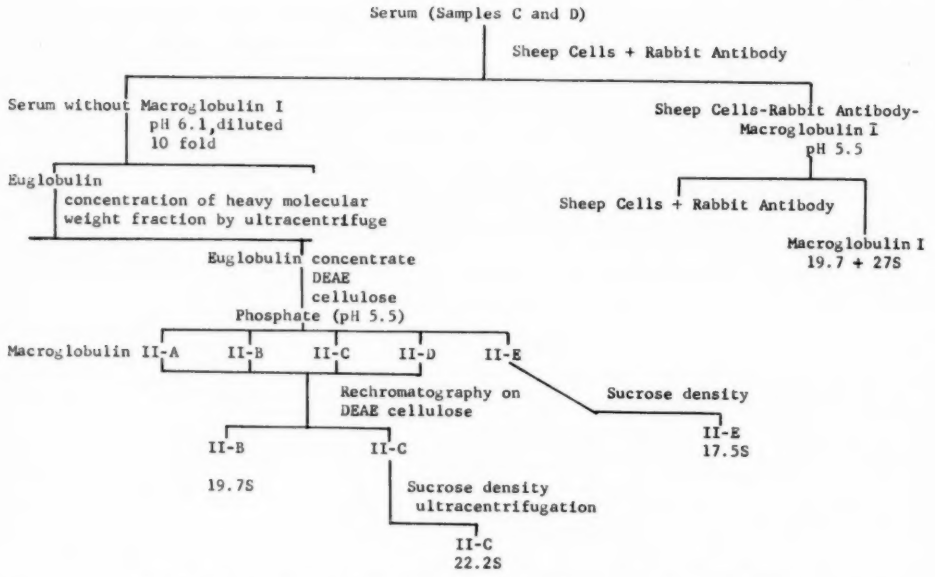


Fig. 1.—Flow sheet of purification of rheumatoid macroglobulins.

antibody* was described previously.[10] In experiments with serum sample C, 330 ml. of packed stroma were sensitized with 165 ml. of homologous rabbit antibody. After thorough washing, the resulting antigen-antibody complex was suspended in 109 ml. of serum. All activity toward the sensitized sheep cell agglutination test[12] was removed by this procedure, but the titer of the serum when tested with reagents that contained human gamma globulin was only moderately reduced. The antigen-antibody–rheumatoid factor complex was then repeatedly washed with cold saline and finally suspended in 382 ml. of phosphate buffer (pH 5.5, 0.15M) to reverse the adsorption of the rheumatoid factor. Five such buffer treatments were required to complete its elution. The pooled opalescent supernatant, spun for 30 minutes at 3,000 r.p.m. to remove stroma particles, was concentrated by ultrafiltration with an ultrafine filter† (very dense porosity, 150 mm. diameter). Concentration of the supernatant to 30 ml. at 4° C. under 15 pounds of nitrogen required nearly one week. During this process small brown particles separated and were discarded. The concentrate was then spun in the ultracentrifuge at 147,090 g for 16 hours, so that all serologically active material was accumulated into a gelatinous brown pellet. This pellet was dissolved in 5 ml. of cold phosphate buffer (pH 5.5, 0.15M) and the resulting opalescent solution was further purified by density gradient ultracentrifugation. In this manner seven fractions were obtained, of which the bottom two contained the greatest amount of serologic activity. These were concentrated and further purified by density gradient ultracentrifugation. A gelatinous but colorless pellet was eventually obtained, which will be referred to in this report as macroglobulin I. Serum sample D was similarly processed.

2. *Purification of macroglobulin II:* The serum fraction which was absorbed with the antigen-antibody complex—now devoid of macroglobulin I and inactive in serologic tests using rabbit antibody—was diluted tenfold with distilled water and adjusted to pH 6.1. After standing in the cold for one hour, the precipitated euglobulin was collected by centrifugation, dissolved in phosphate buffer (pH 7, 0.15M), and reconcentrated by ultracentrifugation. The concentrate was dialyzed against phosphate buffer (pH 7.0, 0.03M), and placed

*Rabbit antisheep cell serum (hemolysin titer 1:3,000) was a gift of Dr. Daniel Widelock, Department of Health, New York, N. Y.

†Carl Schleicher & Schuell Company, Keene, N. H.

on a chromatographic column (20 by 1.5 cm.) of DEAE cellulose,* equilibrated with phosphate buffer of similar pH and ionic strength. The column was eluted with phosphate buffers of decreasing pH but increasing ionic strength at room temperature and at an approximate rate of 5 to 10 drops per minute. The pH and the molarity of the eluting buffers are shown in Figs. 6 and 7. Up to 500 individual fractions, 12 ml. in volume, were collected. The fractions which contained serologically active materials were pooled, as shown in Figs. 6 and 7, and concentrated by ultrafiltration and ultracentrifugation. Concentrates II-B, II-C, and II-E were further purified by density gradient ultracentrifugation. The subfractions with significant serologic activity were pooled, reconcentrated, dialyzed against phosphate buffer (0.15M, pH 5.5), and analyzed by ultracentrifugation.

ANALYTIC METHODS

Protein Analyses.—Protein analyses for starch block electrophoresis experiments were done colorimetrically, using the Folin-Ciocalteu reagent. The progress of the purification by adsorption onto and elution from sensitized sheep erythrocyte stroma and of the fractionation by DEAE cellulose chromatography was followed by spectrophotometric analysis by measurement of the absorption at 278 mμ. In moving boundary electrophoresis, sedimentation analyses, and the determination of the serologic activity, protein concentrations were determined by micro-Kjeldahl digestion followed by Nesslerization.

Serologic Methods.—Serologic activities were estimated by the sensitized (rabbit antibody) sheep cell agglutination test,[12] the potentiated sheep cell (rabbit antibody) agglutination test,[13] Heller's tanned sheep cell (human gamma globulin†) test,[14] and the latex‡ fixation (human gamma globulin) test.[15]

RESULTS

Electrophoresis of Serum Samples.—Moving boundary electrophoresis of serum sample A (Fig. 2)§ with a total protein concentration of 7.5 Gm. per cent gave 54 per cent albumin, 7.4 per cent alpha$_1$ globulin, 19.8 per cent alpha$_2$ globulin, 15.3 per cent beta globulin, and 3.5 per cent gamma globulin. The beta anomaly can be seen in the descending pattern. The ascending pattern was slightly inclined. A slight enantiographic departure with respect to gamma globulin also can be seen. On paper electrophoresis serum sample E (drawn 2 years after sample A) showed no gamma globulin abnormality.

Serologic Data.—Serum sample A had sensitized sheep cell agglutination titer 1:3,500, potentiated sheep cell agglutination titer 1:56,000, Heller's tanned sheep cell agglutination titer 1:112,000, and latex fixation titer 1:112,000. Serum samples B to E inclusive gave similar results in the sensitized sheep cell agglutination and latex fixation tests. Other tests were not performed on these samples.

Starch Block Electrophoresis.—Serum sample B (Fig. 3*A*) was run in diethylbarbiturate buffer at pH 8.6. Positive serologic tests appeared in nearly all globulin fractions. Fraction 2, which represented fast gamma globulin or slow beta globulin, had maximal activity toward cells coated with human gamma

*Diethylaminoethyl cellulose was obtained from Brown Co., Berlin, N. H.

†Human gamma globulin was a gift of the National American Red Cross.

‡Latex particles were provided by Dr. Jacques M. Singer.

§We are indebted to Dr. Eric Pollaczek and Miss M. E. Rivera, Veterans Administration Hospital, Bronx, New York, for performing this analysis.

globulin, whereas fractions 10 to 12 were most active in tests using cells coated with rabbit antibody. Fractions 7 and 8, probably alpha$_2$ globulins, contained material which was active in tests in which human gamma globulin was used, but inactive in tests that required rabbit antibody.

Serum sample E, a fresh serum, run at pH 7.8 phosphate buffer had serologic activity in the fast gamma globulins as well as in the beta globulins. No activity was found in the alpha globulins. A human gamma globulin marker was run concurrently in this experiment.

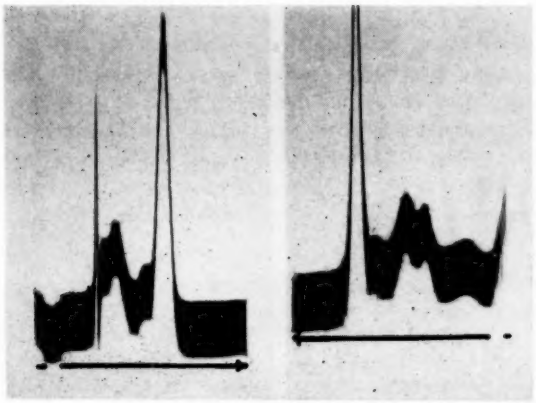
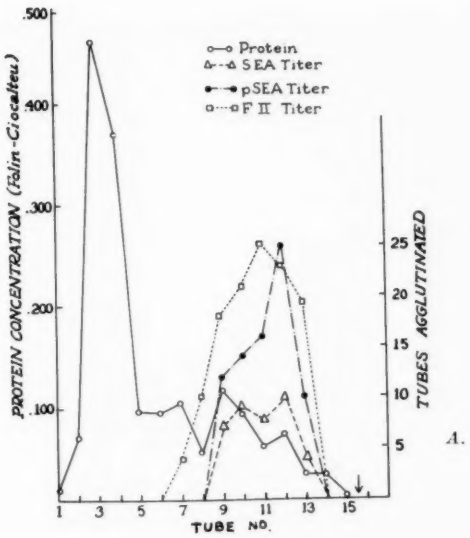


Fig. 2.—Moving boundary electrophoresis pattern of serum sample A. Diethylbarbiturate buffer, pH 8.6, $\mu = 0.1$. Ascending pattern on the right, descending pattern on the left.

Purification of Macroglobulin I.—Macroglobulin I, purified by absorption of sample C or sample D onto and elution from sensitized sheep erythrocyte stroma, was analyzed in the ultracentrifuge (Fig. 4*A*). Both serum samples yielded a two component system, in which the more slowly sedimenting material accounted for 85 per cent of the total concentration. Fig. 5 indicates that on extrapolation to infinite dilution the sedimentation constant of this component was 19.7S. The minor component had a sedimentation coefficient which exceeded 26.7S. Its value was not extrapolated to infinite dilution because of low concentration. The major peak was checked for homogeneity by the area-height method of Faxen.[16] The diffusion coefficient so obtained varied between 3.04×10^{-7} for initial frames to 6.05×10^{-7} cm.^{2} sec^{-1} for later frames. A homogeneous material should have given a reasonably constant diffusion coefficient. An increase of this coefficient as a function of time in sedimentation experiments indicates inhomogeneity.

Since in our experience prolonged dialysis in pH 8.6 diethylbarbiturate buffer resulted in considerable precipitation and degradation of the macroglobulin, moving boundary electrophoresis of macroglobulin I was carried out in phosphate buffer at pH 7.0 ($\mu = 0.45$). Two components were found (Fig. 4*B*). One peak, comprising 85 per cent of the material, had a mobility of -2.2×10^{-5}



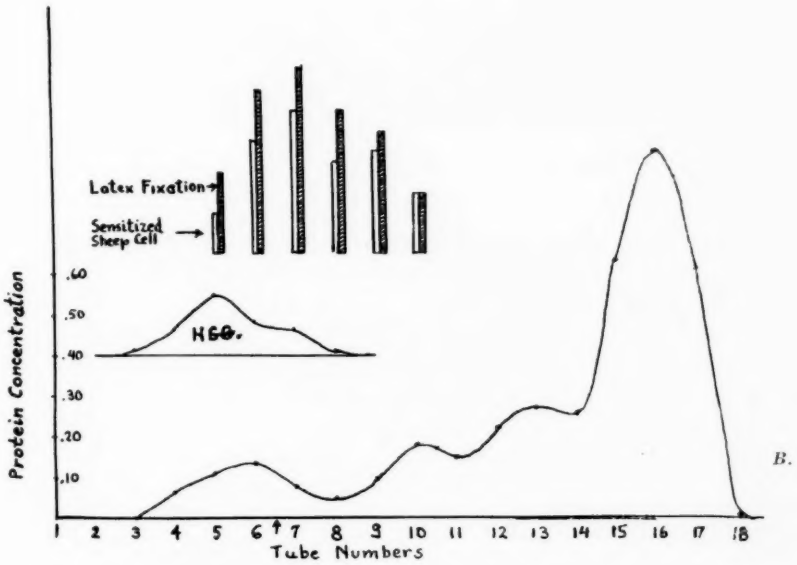


Fig. 3.—*A*, Starch block electrophoresis of serum sample B. Diethylbarbiturate-saline buffer, pH 8.6, $\mu = 0.1$. *SEA*, Sensitized sheep cell agglutination; *pSEA*, potentiated sheep cell agglutination; *F II*, Heller's tanned sheep cell agglutination. *B*, Starch block electrophoresis of serum sample E in pH 7.6 phosphate buffer ($\mu = 0.2$). *HGG*, Human gamma globulin marker. Activity appears in tube numbers 5 to 10.

cm.^{2} volt^{-1} sec^{-1}; the minor peak migrated at -4.2×10^{-5} cm.^{2} volt^{-1} sec^{-1}. Under comparable conditions of pH, ionic strength, and concentration a sample of human gamma globulin had a mobility of -0.35×10^{-7} cm.^{2} volt^{-1} sec^{-1} at the maximal ordinate.*

*We wish to thank Mr. Thomas Hayes, College of Physicians and Surgeons, Columbia University, for assisting in this experiment.

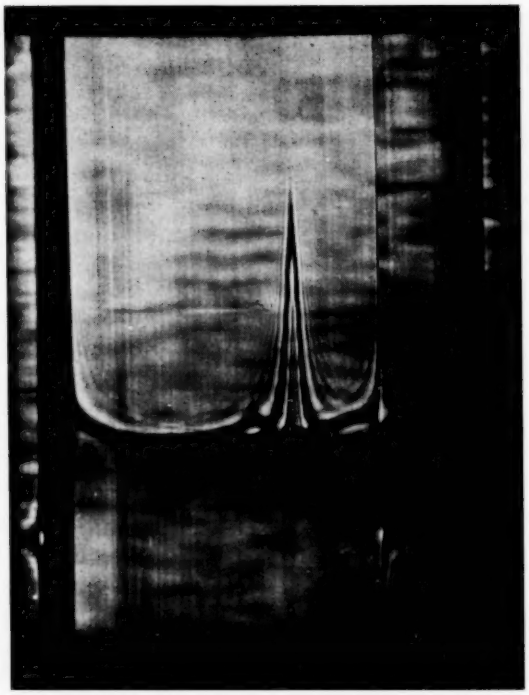


Fig. 4*A*.—Ultracentrifuge pattern of macroglobulin I at 16 minutes, 47,660 r.p.m.

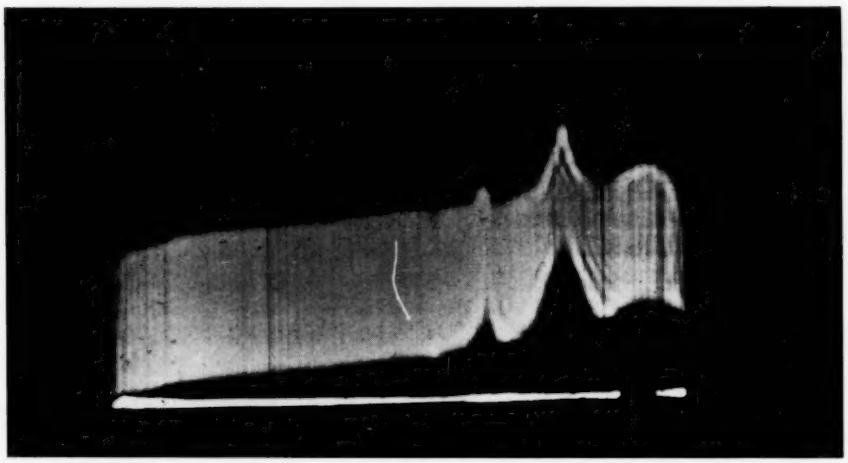


Fig. 4*B*.—Ascending electrophoresis pattern of macroglobulin I in pH 7 phosphate buffer ($\mu = 0.45$).

The serologic activity of macroglobulin I was as follows: at the end point of titration the protein concentration in the sensitized sheep cell agglutination test was 0.017 meg. per milliliter, and 0.034 meg. per milliliter in the latex

particle fixation test. The yield of macroglobulin I was approximately 6 mg. The theoretical yield (based on a 1:3,500 titer in the sensitized sheep cell test) is also approximately 6 mg.

Purification of Macroglobulin II.—A diagram of the elution pattern of the anion exchange chromatography with DEAE cellulose is shown in Fig. 6 (serum sample C). The number of fractions collected as well as the molarity and pH of the eluting buffers are given on the abscissa. The protein concentration and the latex fixation titer of the eluates are plotted on the ordinate. Three major areas of latex fixation were found, namely, on elution with 0.10M phosphate buffer (pH 5.5), with 0.15M phosphate buffer (pH 5.5), and with 0.15M phosphate buffer (pH 5.5) to which 0.1M sodium chloride had been added. The

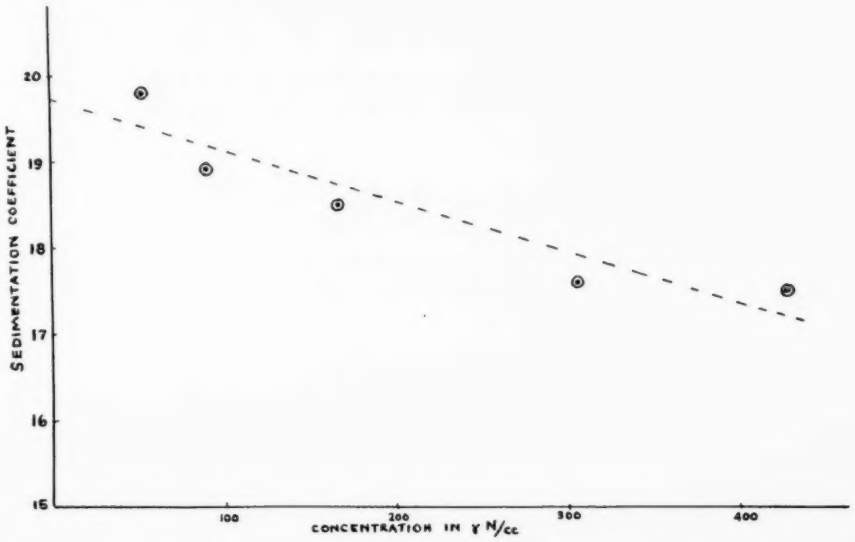


Fig. 5.—Dependence of the sedimentation coefficient of macroglobulin I on concentration.

serologically active concentrates II-A, II-B, II-C, II-D, and II-E were analyzed in the ultracentrifuge. II-A, with a very moderate latex fixation activity, contained only negligible amounts of macroglobulin. II-B was a single component macroglobulin. II-C contained three components. The most rapidly sedimenting component comprised 60 per cent, and a component of intermediate sedimentation velocity accounted for 30 per cent of this fraction. Ten per cent of the fraction was 7S. II-D was devoid of significant amounts of macroglobulin, but also showed only weak serologic activity. Macroglobulin II-E contained only a single component of high sedimentation velocity; however, 7S material was present.

Such quantities of fractions II-A to II-D as remained after ultracentrifuge analysis were pooled, dialyzed against 0.03M phosphate buffer, pH 7.0, and reapplied on another DEAE cellulose column. The purpose of the pooling was

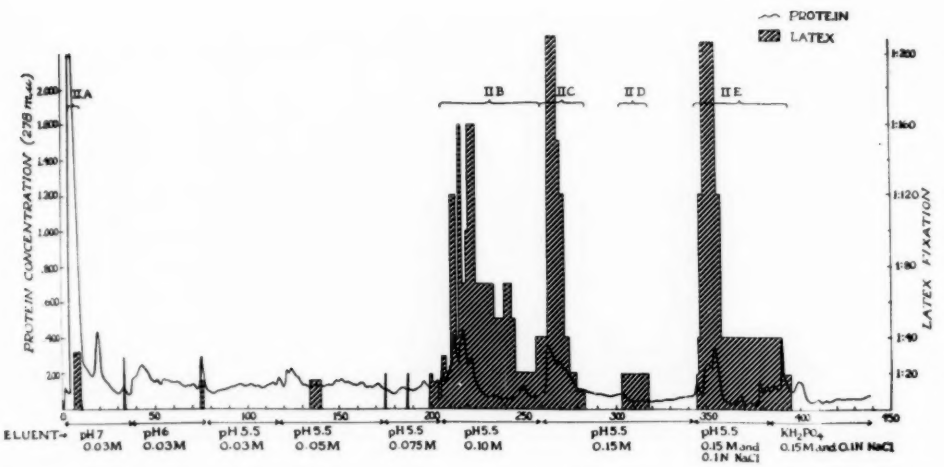


Fig. 6.—DEAE cellulose chromatography of serum fraction containing macroglobulin II. Shaded areas indicate latex fixation activity of eluate. The braces indicate the extent of pooling for each serologically active fraction. The tube numbers and the types of eluent used are indicated in the abscissa.

to ascertain whether repetition of the elution process would result in a reasonably identical elution pattern, or whether the results obtained on column chromotography were artifacts. Fraction II-E was omitted in the repeat elution in order to see whether a similar material might appear. The serologically active material eluted at pH 5.5, 0.10 and 0.15M (Fig. 7). Tubes which corresponded to those comprising II-E in the previous elution experiment contained only negligible amounts of protein and little serologic activity. Chromatography of these macroglobulins on DEAE cellulose appears, therefore, to be reproducible. II-B in the ultracentrifuge was, as before, a single component system with an approximate sedimentation coefficient of 19S. II-C, however, mainly contained a rapidly sedimenting component, whereas originally 40 per cent of other material was present.

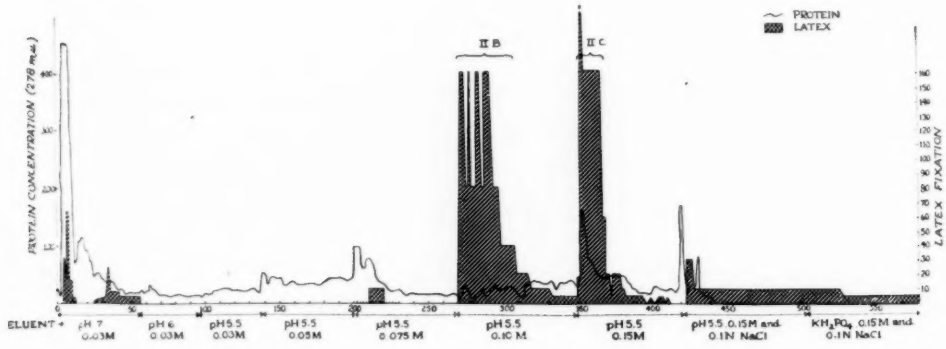


Fig. 7.—DEAE cellulose chromatography of fractions II-A, II-B, II-C, and II-D. Shaded areas indicate latex fixation activity in eluate. The tube numbers and the types of eluent used are indicated in the abscissa.

The following are the results of the purification of serum sample D: insignificant amounts of macroglobulin were found in the first ten tubes (II-A). II-B contained 30 per cent 7S and 70 per cent of what appeared to be a 19S material. II-C, as in serum sample C, was a mixture of three components. The major component, sedimenting fastest, was isolated by sucrose density gradient purification. II-D contained only traces of macroglobulin, and II-E, as in serum sample C, contained a single macroglobulin. On ultracentrifugation at three different concentrations, purified macroglobulin II-C had a sedimentation constant of 22.2S (solid line, Fig. 8). Purified macroglobulin II-E had a sedimentation constant of 17.5S (dotted line, Fig. 8). A sedimentation constant of the purified macroglobulin of fraction II-B was not obtained, but on the basis of a single sedimentation velocity experiment, a tentative S value of 19.7 was assigned to it. Reproductions of the sedimentation patterns of the purified macroglobulins of II-B, II-C, and II-E appear in Fig. 9. Calculation of the diffusion coefficient of macroglobulin II-C gave 2.6×10^{-7} cm.^{2} sec^{-1} for the first two frames; frames 3, 4, and 5, however, gave diffusion coefficients up to 3.5×10^{-7} cm.^{2} sec^{-1}. This indicates that this macroglobulin was not completely homogeneous.

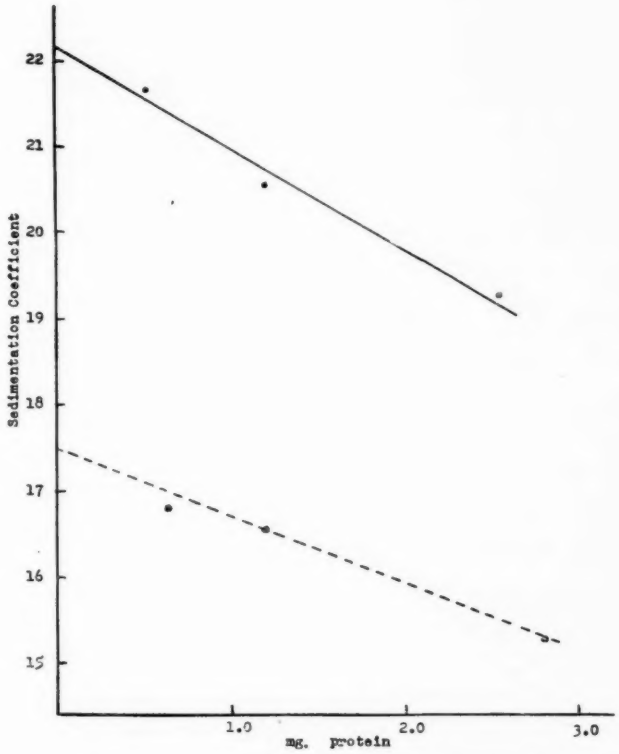
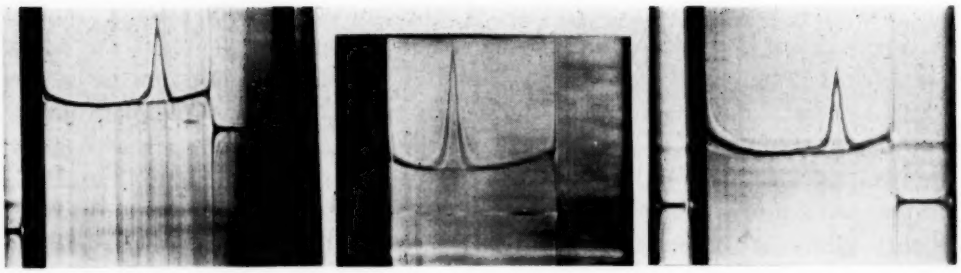
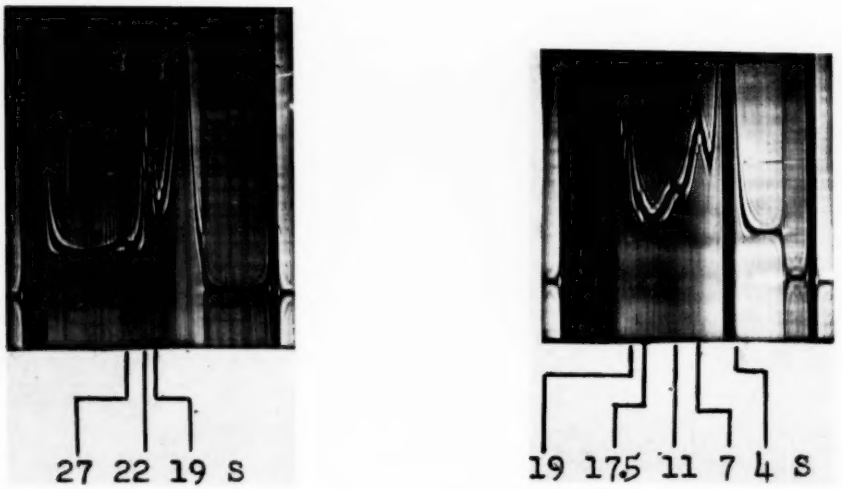


Fig. 8.—Dependence of the sedimentation coefficients of macroglobulin II-C (solid line) and of macroglobulin II-E (dotted line) on concentration.



A. *B.* *C.*

Fig. 9.—Sedimentation patterns of *A*, macroglobulin II-B (at 16 minutes), *B*, macroglobulin II-C (at 24 minutes), and *C*, macroglobulin II-E (at 16 minutes), on ultracentrifugation at 47,660 r.p.m.



A. *B.*

Fig. 10.—Ultracentrifuge patterns of a macroglobular concentrate of serum sample E. *A*, Pattern after 16 minutes at 59,760 r.p.m. showing 27S, 22S, 19.7S macroglobulins. *B*, Pattern of a more concentrated sample after 64 minutes at 59,760 r.p.m., showing the 19.7S, 17.5S, 11S, 7S and 4S components. The 27S and 22S components have already disappeared.

All components of macroglobulin II were inactive in the sensitized sheep cell test, but 0.25 (±0.05) meg. per milliliter of macroglobulin II-B, II-C, and II-E still reacted with latex particles coated with human gamma globulin. No significant differences were found in specific activity of the latex fixation test between the various components of macroglobulin II.

Ultracentrifugation of a Serum Sample.—Serum sample E, at 59,780 r.p.m., showed two shallow peaks, probably corresponding to the 19S and the 22S components. When 6 ml. of sample E was concentrated by ultracentrifugation into a pellet, and analyzed when redissolved in saline, four macroglobular peaks were seen. Fig. 10, *A* shows the ultracentrifuge pattern of this concentrate, with its rapidly moving component, probably exceeding 27S, and hypersharp 22S and 19S components. Because of the magnitude of the 19S peak, the 17.5S material

cannot be seen. Using a more concentrated preparation, on prolonged ultracentrifugation a shallow 17.5S component could be seen to separate from the dominant 19S peak (Fig. 10, *B*). Sera of other individuals were also analyzed in a similar fashion. A fresh normal serum showed only a 19S component. Samples of normal sera which were pooled and kept in a freezer for approximately one year gave a minute elevation at approximately 27S. The serum of an individual with hemochromatosis showed only a 19S component. In three rheumatoid sera with high serologic activity, no 27S or 22S components were seen. One other rheumatoid serum, however, exhibited a small 22S but no 27S component. One serum sample from still another patient with rheumatoid arthritis contained large 19S and 22S components, and also a small amount of 27S. No 17.5S component was encountered in the other serum samples tested. These results are summarized in Table I.

DISCUSSION

Different samples of this individual's serum, drawn during a 2 year period, were studied. Although serum sample A showed low values of gamma globulin, no other quantitative electrophoretic analyses of serum were performed until sample E, which was drawn approximately 2 years after sample A. Paper electrophoresis of sample E, which was not stored in the freezer, gave a gamma globulin value within the lower limit of normality. The low gamma globulin level of serum sample A, though not correlated with frequent occurrences of infection, might have been related to steroid therapy.

Zone electrophoresis of sample B showed a wide distribution of serologically active material. Of particular interest was the fact that activity toward particles sensitized with human gamma globulin was found in more fractions than activity toward cells sensitized with rabbit antibody. Sample E was run at pH 7.6 in order to effect a better separation of the gamma and beta globulins. In this experiment a gamma globulin marker was run concurrently. The serologic activity of sample E was found in the gamma and beta globulins only; there was no evidence of the presence of serologic activity in the alpha globulins. The discrepancies in the results of electrophoretic studies of serum samples B and E might be attributable to three possibilities: (a) serum sample B antedated sample E by nearly 2 years; (b) serum sample B was kept in the freezer for a considerable period of time, whereas sample E was a freshly drawn sample which had not been frozen; and (c) changes in the characteristics of the rheumatoid macroglobulins could have occurred during this 2 year interval.

In contrast to the previously reported experience with other serum preparations,[10] on ultracentrifugation macroglobulin I in addition to the usual 19S component contained small amounts of rapidly sedimenting material. Electrophoretic analysis of macroglobulin I also showed two distinct components, whose mobility exceeded that of normal human gamma globulin. The 27S material was not obtained free of the dominant 19.7S material, but that it is indeed a constituent of the rheumatoid factor is suggested by the following facts. It

TABLE I. OCCURRENCE OF MACROGLOBULINS IN SERUM CONCENTRATES

TYPE OF SERUM	17S	19S	22S	27S
Normal fresh serum	–	++	–	–
Pooled normal sera	–	++	–	±
Hemochromatosis	–	++	–	–
Rheumatoid arthritis	–	++	–	–
Rheumatoid arthritis	–	++	–	–
Rheumatoid arthritis	–	++	–	–
Rheumatoid arthritis	–	++	+	–
Rheumatoid arthritis	–	++	++	+
Rheumatoid arthritis	+	++	++	+

was absorbed onto and eluted from sensitized stroma together with the 19.7S material. Macroglobulins are invariably absent in similarly processed control sera. The 27S component, however, was absent in five serum samples of individuals with rheumatoid arthritis that had been previously purified by this procedure.[10] This might have been due to the rarity and/or low concentration of this component. The 27S component was part of macroglobulin I in samples C and D, and in each experiment appeared in a quantitative ratio of 1:6 with respect to the major component. A component of similar sedimentation velocity has been demonstrated on analytic ultracentrifugation of a concentrate of this individual's serum, and therefore is probably not the result of manipulation. The 27S component could also be removed from this individual's serum by a complex of rabbit antihuman serum and human gamma globulin. It thus appears that the 27S component may have escaped detection until now because of its low concentration and rare occurrence. In the latter respect it somewhat resembles the 22S component, which, according to Kunkel and his associates,[21] appears in approximately 30 per cent of sera of individuals with rheumatoid arthritis. Further work on the characterization of this component, its occurrence in other sera, and its behavior toward urea is now in progress.

In this individual's serum, macroglobulin II was a mixture of at least three well-defined components. The fractions obtained by chromatography, namely II-A to II-D inclusively, were rerun on an identical chromatographic column in order to test the reproducibility of this type of purification. The results of these experiments indicate that the order of elution of the macroglobulins cannot be attributed to experimental artifacts. The 17.5S macroglobulin, which appears to be a rare and minor component of the rheumatoid factor, has not been described previously. The 22.2S macroglobulin has not been previously isolated. A macroglobulin of this sedimentation velocity has been shown by Kunkel and his associates[21] to be part of the rheumatoid factor, and this component was dissociated by them into serologically active 19S and serologically inactive 7S constituents. These investigators, however, have not described its serologic specificity, because it was not obtained free of 19S macroglobulin. It is of note that these investigators have found a poor correlation between the height of the 22S peak and the sensitized sheep cell activity of sera that contain such 22S material. Whether the 22.2S component is thus exclusively part of macroglobulin II is, however, subject to further investigation.

In view of the small amounts of purified macroglobulins that remain after sedimentation analysis and the time elapsed between the preparation and the analyses, it was not possible to recombine the isolated macroglobulins in order to test their heterogeneity in the ultracentrifuge. On ultracentrifugation a concentrated macroglobular preparation of this patient's serum contained four macroglobular components. This result is compatible with the fractionation experiments. The 17.5S material was present in small quantities and could only be resolved with some difficulty from the dominant 19.7S component. The amount of 27S macroglobulin is also small, and this corresponds well to the relative concentration of the 27S component in macroglobulin I. It would appear that the 19S and 22S macroglobulins are the dominant components of the rheumatoid factor, and that the 17.5S and 27S macroglobulins are minor and probably rare constituents. No attempts were made to degrade the various macroglobulins with urea, but such studies are now in progress. At the present time it is uncertain whether all rheumatoid factors are ultimately reducible to a single parent compound.

The degree of purity of some of the isolated macroglobulins was investigated by calculation of their diffusion constants. In view of the limited amounts of material available, such data had to be obtained from sedimentation velocity experiments alone, although it was realized that Schlieren optics do not yield optimal results. Although the 19.7S peak of macroglobulin I, and the 22.2S peak of macroglobulin II-C appeared bilaterally symmetrical, their calculated diffusion constants suggest heterogeneity. In a previous publication,[10] however, a rheumatoid factor was described, having a diffusion constant of 2×10^{-7} cm.^{2} sec^{-1}, which satisfied the homogeneity criteria of the Faxen equation.

There is no doubt that macroglobulin I is different from macroglobulin II serologically and, therefore, structurally. The latter fails to react with carrier cells sensitized with rabbit antibody, and its specific reactivity toward systems that contain human gamma globulin is one tenth of that observed with macroglobulin I. Reasons for this difference are not known. Dilute sera, that contained low titers of rheumatoid factor, were added to preparations of macroglobulin II, but failed to potentiate the titers. Sheep serum also failed to potentiate. Therefore, it is unlikely that these macroglobulins require a cofactor for maximal interaction with the gamma globulin reactants.

Unfortunately, insufficient quantities of the various members of macroglobulin II were available for moving boundary electrophoresis, but the starch block electrophoresis experiments of the serum sample indicate that the two types of macroglobulin have reasonably similar mobilities.

Evidence has been provided that one individual's serum may contain at least five distinct macroglobulins, namely, the 27S and 19.7S macroglobulin I, and the 17.5S, 19.7S, and 22.2S macroglobulin II. Fractionation of sera of other individuals with rheumatoid arthritis has also given evidence of heterogeneity of rheumatoid factors.[20] A comprehensive report on this subject is in preparation.

Heterogeneous rheumatoid factors have also been found by other investigators. On DEAE cellulose chromatography of a dissociated rheumatoid factor–gamma globulin complex, Lospalluto and Ziff[4] obtained two fractions of differing serologic behavior. Williams and co-workers[5] have found a minor 30S component in their rheumatoid factor preparation. Whether this high molecular weight component has the serologic activity of the rheumatoid factors is not certain.

The findings presented in this report shed new light on the possible nature of the rheumatoid factors. Because of the presence of a family of proteins of high sedimentation coefficient, the rheumatoid factors resemble the macroglobulins that occur in Waldenström's macroglobulinemia, which in most instances are gamma globulins.[17] Patient specificity with respect to the synthesis of macroglobulins occurs in Waldenström's macroglobulinemia[18] and probably also in rheumatoid arthritis. However, in contrast to Waldenström's macroglobulinemia, only small quantities of macroglobulin are found in rheumatoid arthritis, and no instances are known in which the elaboration of the rheumatoid factors interfered with the synthesis of other antibodies.

On the other hand, one cannot ignore the tempting hypothesis that the rheumatoid factors are antibodies.[2, 22] In support of this, one might even cite the existence of closely related macroglobulins. The occasional appearance of similar macroglobulins in diseases other than rheumatoid arthritis does not vitiate such a hypothesis, for common reactivity with serologic reagents cannot be taken as proof that the compounds present in the sera of such patients are identical with the rheumatoid factors.

One might, however, wonder whether five species of macroglobulin, varying from 17.5S to 27S, could really cross react with aggregated human gamma globulin or whether perhaps these reactions are in reality only due to protein-protein interactions.

SUMMARY

Fractionation of serum samples of an individual with classic rheumatoid arthritis and a transient hypogammaglobulinemia without concurrent frequent infections is reported. By absorption onto an antigen-antibody complex and its elution therefrom, two proteins of the rheumatoid factor family were isolated (macroglobulin I). Macroglobulin I contributed most heavily to the serum's serologic reactivity with carriers coated with human gamma globulin and possessed the sole quality of reacting with cells sensitized with rabbit antibody. The proteins had sedimentation constants of 19.7S and 27S, respectively. On electrophoresis in pH 7 phosphate buffer the mobilities were substantially higher than that of human gamma globulin. After removal of macroglobulin I, three further members of the rheumatoid factor family were isolated by means of DEAE cellulose chromatography. These were called macroglobulin II, with sedimentation constants of 17.5S, 22.2S, and probably 19.7S. Macroglobulin II failed to react with cells sensitized with rabbit antibody. In serologic tests in which carriers coated with human gamma globulin were employed macroglobulin II possessed one tenth of the activity of macroglobulin I. Therefore, a total of five distinct macroglobulins with the characteristics of the rheumatoid factor

were found in this individual's serum. The results of the purification experiments were verified by analysis in the ultracentrifuge of a concentrate of fresh serum. The expected four component macroglobular system was seen in such a preparation. The significance of these findings is discussed.

Acknowledgment is made to Dr. Mary L. Petermann and Dr. Leonhard Korngold for advice.

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RED CELL ELECTROLYTES IN PATIENTS WITH EDEMA

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This investigation was undertaken to determine the pattern of electrolyte and water concentration in the erythrocytes of edematous patients in order to ascertain differences in hyponatremic and normonatremic individuals, and to learn what differences exist during different phases of therapy. Fifty-six determinations were made in thirty-one edematous patients with renal disease, cirrhosis with ascites, and congestive heart failure. The results during the accumulation of edema or when there was therapeutic unresponsiveness were compared to twenty-four normal subjects. The erythrocyte concentration of sodium was decreased and water was increased. Erythrocyte potassium concentration was not changed significantly, which suggested an increase in total potassium content. The sodium concentration and water content were the same in both hyponatremic and normonatremic patients, but erythrocyte potassium concentration was decreased significantly in hyponatremic patients when compared to normonatremic edematous individuals. During diuresis erythrocyte sodium concentration increased and again declined as diuresis ceased. It was suggested that osmotic and pH effects alone did not explain the changes observed, and that the movement of sodium out of the erythrocytes during the development of edema may be related to increased aldosterone activity.

PATIENTS with edema exhibit a generalized expansion of body fluid volume. Total exchangeable body sodium is increased and sodium space is expanded.[1-4] Observations and calculations based on metabolic balances have been interpreted to indicate that sodium assumes an intracellular position during diuresis, and, conversely, sodium is considered to leave the cells during edema accumulation.[5-9] In these circumstances sodium and potassium may move in the same direction during the accumulation and loss of edema fluid.[7, 8, 10] However, measurements of muscle sodium have failed to reveal an increase in sodium content in many subjects during the development of or recovery from congestive failure or cirrhosis with ascites.[10-12]

Although patients with edema and hyponatremia exhibit increased exchangeable body sodium[4, 13, 14] and increased total body water, the question of the contribution of increased intracellular sodium content to the hyponatremia is not entirely answered. It is certain, however, that the hyponatremia of edematous

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patients is the result of several factors,[14] only one of which may be the intracellular migration of sodium.

The erythrocytes have been shown to take part in many generalized aberrations of electrolyte metabolism, including edema states.[15-24] The results obtained in edema states, however, have been variable.[22-24] The investigation to be reported was designed (1) to obtain further information regarding the electrolyte patterns of the red cells in edematous patients, (2) to offer an explanation for the previously reported variations in results, (3) to assess the role of intracellular migration of sodium as a factor in the production of hyponatremia in edematous patients using the erythrocytes as a model, and (4) to gain insight into some of the factors contributing to red cell electrolyte concentration in edematous subjects.

MATERIAL AND METHODS

This investigation represents 56 observations on thirty-one patients with edema due to various causes. Seventeen observations were made in patients with renal disease of various types including acute and chronic renal failure, a number of whom also had heart failure, the nephrotic syndrome, and one patient with a renal carcinoma. Twenty-one observations were made in patients with cirrhosis with ascites, and 18 observations were made in patients with congestive heart failure due to cor pulmonale, rheumatic heart disease, and arteriosclerotic heart disease. When possible, patients were studied while edema was accumulating or while their condition was static, during a diuresis, and after cessation of a diuresis. All patients except those with severe renal insufficiency were on diets containing 20 mEq. of sodium daily, and patients with congestive heart failure had previously been digitalized. The patients were compared to twenty-four individuals without evidence of cardiovascular, renal, hepatic, or endocrine disorders. Samples of blood were drawn into siliconized syringes without venous stasis and delivered into flasks that contained powdered ammonium heparin as an anticoagulant. Assay of the ammonium heparin showed an absence of sodium and potassium. All analyses were performed in duplicate. The concentrations of sodium and potassium in plasma and laked whole blood were determined in a flame photometer using lithium as an internal standard. Recovery studies indicated an accuracy for sodium in whole blood of ± 1.0 mEq. per liter, in plasma of ± 2.0 mEq. per liter, for potassium in whole blood ± 0.56 mEq. per liter, and in plasma ± 0.1 mEq. per liter. For sodium when duplicate determinations on whole blood did not agree to within 1 mEq. per liter additional analyses were performed in duplicate. In such instances the average of three results was taken if the replicate analyses agreed to within 1 mEq. per liter or else the determination was discarded. The same procedure was followed in the analysis for plasma sodium and whole blood and plasma potassium, using limits of agreement in the duplicates of 2.0 mEq. per liter, 0.56 mEq. per liter, and 0.1 mEq. per liter, respectively. Chloride was determined in plasma and in whole blood, after sodium tungstate and sulfuric acid precipitation, by the method of Shales and Shales.[25] This precipitation of whole blood did not affect the chloride determination. Water content of plasma and whole blood was determined from the difference in weight before and after drying in a drying oven at 105° C. for 48 hours. This was found to be adequate to bring the specimens to a constant weight. The limits of tolerance between duplicates found acceptable for the calculation of red cell water were 45 Gm. per liter of cells for both whole blood and plasma. Hematocrit was measured in Wintrobe tubes spun in a centrifuge for 45 minutes at 1,000 g. Duplicates agreed within 0.5 per cent. The erythrocyte concentrations of sodium, potassium, chloride, and water were calculated according to formulas used in a previous investigation.[26]

In analyzing the data the various groups were broken down into several categories and statistical analyses were made by the methods described by Snedecor.[27]

RESULTS

The results for the individual categories of patients studied and for the entire group are given in Table I. Edematous subjects with renal disease, Laennec's cirrhosis, and congestive heart failure exhibited significant reductions in erythrocyte sodium concentration and increases in erythrocyte water content. The erythrocyte concentrations of potassium were essentially unchanged and chloride was not significantly reduced when the entire group was compared to normal subjects. Patients with congestive heart failure tended to show an increase in erythrocyte potassium concentration, but this was not statistically significant.

TABLE I. COMPARISON OF RED BLOOD CELL ELECTROLYTES AND WATER IN NORMAL AND EDEMATOUS PATIENTS WITH RENAL DISEASE, CIRRHOSIS WITH ASCITES AND CONGESTIVE HEART FAILURE

SUBSTANCE	SUBJECTS	NO. OF DETERMINATIONS	MEAN	RANGE	S.D.	T	P
Na	Normal	24	17.6	7.0-24.2	4.7		
mEq./Kg	Renal	17	7.6	0.0-20.1	7.2	5.39	< 0.001
cell H_2O	Cirrhosis	21	11.2	3.0-25.6	7.2	3.67	< 0.01
	Congestive failure	18	10.6	0.0-19.9	6.3	4.14	< 0.001
	All subjects	56	10.1	0.0-25.6	7.0	4.81	< 0.001
K	Normal	24	135	119-146	5.8		
mEq/Kg	Renal	17	135	110-171	14.3	---	
cell H_2O	Cirrhosis	21	137	114-151	9.8	0.86	> 0.1
	Congestive failure	18	140	112-154	11.9	1.80	> 0.05
	All subjects	56	137	110-171	11.8	0.79	> 0.1
Cl	Normal	24	89	57-118	19.1		
mEq/Kg	Renal	17	87	35-139	27.1	0.87	> 0.1
cell H_2O	Cirrhosis	21	86	54-108	15.0	0.62	> 0.1
	Congestive failure	18	82	57-110	14.0	1.31	< 0.1
	All subjects	56	83	35-139	18.9	1.30	< 0.1
H_2O	Normal	24	713	684-727	10.0		
Gm./L.	Renal	17	728	701-770	22.3	2.92	< 0.02
cells	Cirrhosis	21	730	691-762	17.1	4.22	< 0.001
	Congestive failure	18	734	680-766	20.4	4.34	< 0.001
	All subjects	56	730	684-770	19.9	3.55	< 0.01

Because the variation in the group as a whole was wide, the patients were subdivided into three groups according to whether they were (1) actively retaining edema fluid and/or not responding to therapy (33 determinations), (2) were undergoing diuresis or had less severe edema (12 determinations), and (3) immediately after the cessation of a diuresis (11 determinations). The latter does not imply that the subjects were edema-free, for, by and large, edema was still present. The results are given in Fig. 1 and indicate that the erythrocyte sodium concentration, when the patients were not responding to therapy and/or had more severe edema, was significantly depressed to 8.1 ± 5.6 mEq. per kilogram cell water ($p < 0.001$). During diuresis or when edema was less severe the erythrocyte sodium concentration (16.5 ± 7.4 mEq. per kilogram cell water) was not significantly different from normal. Upon cessation of a diuresis the

erythrocyte sodium concentration again declined to 9.2 ± 6.9 mEq. per kilogram cell water. No significant changes occurred in the erythrocyte potassium or chloride concentrations during these phases. Erythrocyte water content was increased before and during diuresis, but decreased after diuresis ceased and was not significantly different from that of normal subjects. When compared to diuresing patients, however, the decline in red cell water content after diuresis ceased was not statistically significant, possibly because the number of individuals in these groups was too small for adequate analysis.

In Fig. 2 the changes in erythrocyte sodium concentration are given in those subjects studied during various phases of edema. In ten of the eleven patients charted the higher erythrocyte sodium concentration during a diuresis or with milder edema is clearly evident, as is the subsequent decline in erythrocyte sodium concentration as diuresis ceased.

Red Blood Cell Electrolytes in Hyponatremic Patients.—Twenty-one determinations were made in hyponatremic patients (plasma sodium concentration range, 110 to 135 mEq. per liter). The erythrocyte sodium, potassium, and water contents are given in Fig. 3, and compared with the concentration of these moieties in thirty-five normonatremic edematous subjects and with twenty-four normal subjects. The results indicate that the erythrocyte sodium concentration is reduced significantly in both hypo- and normonatremic subjects to about the same degree when compared to normal subjects ($p < 0.01$ for both groups). The erythrocyte potassium concentration is not significantly decreased in hyponatremic patients ($p > 0.05$) but is increased slightly in normonatremic patients ($p < 0.05$). When the hyponatremic and normonatremic patients are compared, however, the difference in potassium concentration in the erythrocytes is highly significant (131 ± 12.3 and 141 ± 10.0 mEq.

TABLE II. ERYTHROCYTE WATER CONTENTS IN NORMAL SUBJECTS, NORMONATREMIC, AND HYPONATREMIC PATIENTS WITH EDEMA (GM./L. CELLS)

	NORMAL	NORMONATREMIC	HYPONATREMIC
No. of determinations	24	35	21
Mean	713	729	736
Range	684-727	680-763	691-770
S.D.	10.0	18.3	23.3
t		3.90	4.40
p		< 0.005	< 0.001

TABLE III. CONCENTRATION OF SODIUM PLUS POTASSIUM IN HYPONATREMIC PATIENTS, COMPARED TO NORMONATREMIC PATIENTS WITH EDEMA AND NORMAL SUBJECTS (MEQ./KG. CELL WATER)

	HYPONATREMIC	NORMONATREMIC	NORMAL
No. of determinations	21	35	24
Mean	142	151	152
Range	110-161	132-174	140-164
S.D.	12.4	9.7	6.9
t		3.03	3.50
p		< 0.01	< 0.01

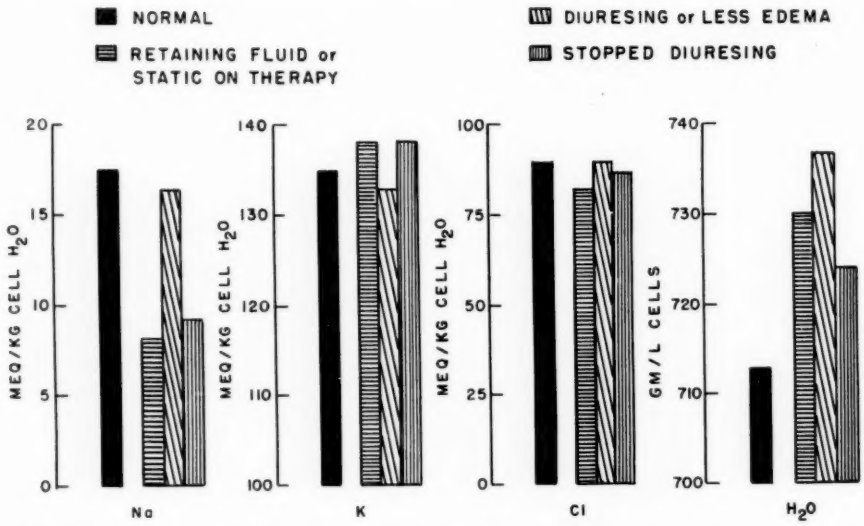

Fig. 1.—Mean red blood cell concentrations of sodium, potassium, chloride, and water in 33 determinations on subjects with edema actively retaining fluid or not responding to therapy, 12 determinations during diuresis or with less severe edema, and 11 determinations after cessation of a diuresis. This is contrasted to the mean values obtained in twenty-four normal subjects.

per kilogram cell water respectively, $p < 0.01$). No significant differences in erythrocyte water were observed between the hyponatremic and normonatremic patients, but both groups had an increased water content when compared to normal subjects (Fig. 3 and Table II).

Although the erythrocyte concentration of potassium was not significantly reduced in hyponatremic patients when compared to normal subjects, it was observed that the sum of the concentration of the cations (sodium plus potassium) was significantly lower in hyponatremic patients than in both normonatremic and normal subjects (Table III).

DISCUSSION

The data presented indicate that in edematous subjects the concentration of sodium in the erythrocytes is reduced and the water content is increased. Since the concentration of potassium in the group as a whole is unchanged, it may be deduced that as the water content of the cells increased the total potassium content increased. When the patient is undergoing diuresis sodium enters the cells, and the concentration may reach supranormal levels. With cessation of the diuresis sodium again leaves. This interpretation is strengthened by the fact that the increased erythrocyte water content did not change during diuresis and decreased, if anything, after the diuresis ceased. After diuresis ceased had sodium remained in the erythrocytes the concentration should have been unchanged or perhaps risen.

The results in the present investigation are somewhat different from those of other investigators who have reported that the concentration of

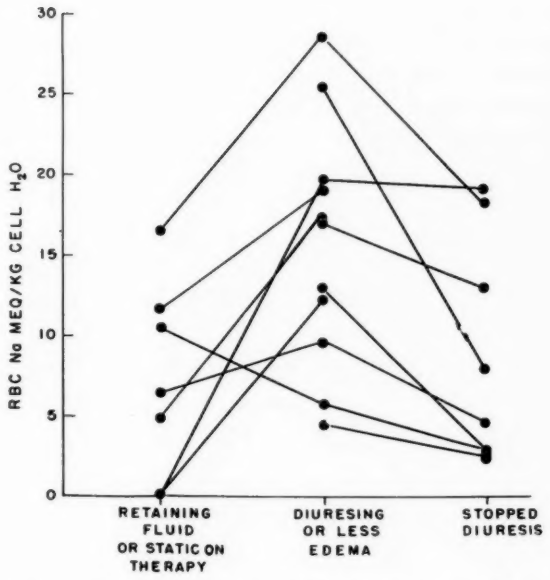

Fig. 2.—Alteration in red blood cell concentration of sodium in subjects studied before, during, and after cessation of a diuresis.

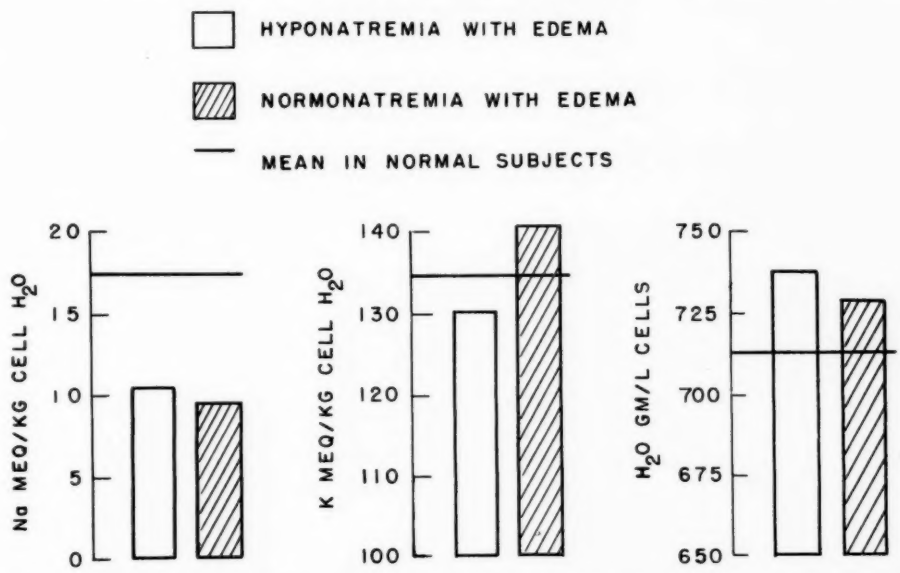

Fig. 3.—Mean red blood cell concentrations in 21 and 35 determinations in subjects with hypo- and normonatremia, respectively, contrasted with 24 normal subjects.

sodium in the erythrocytes in congestive heart failure is increased[22, 24] or not altered.[23] Erythrocyte sodium concentrations were supranormal in several in several of our patients studied during diuresis. Were the investigation weighted with diuresing patients it is distinctly possible that the mean sodium concentration of the erythrocytes would have been normal or increased. It would appear.

therefore, that the results may be influenced by the presence or absence of diuresis at the time of study. In this light, normal, increased, or depressed erythrocyte sodium concentrations may be observed depending upon the proportion of diuresing and nondiuresing patients. In the present investigation the group is heavily weighted with patients actively retaining edema fluid. The heterogeneity of the group of patients studied would also help to explain the greater standard deviations when contrasted to the more homogenous group of normal subjects.

The erythrocyte concentration of sodium and water was essentially the same in both hypo- and normonatremic individuals, suggesting that the intracellular migration of sodium does not contribute to the development of hyponatremia in edematous patients so far as the red cells might reflect changes in other tissue cells. However, the potassium concentrations in the hypo- and normonatremic groups were significantly different. This observation would tend to indicate that the cellular osmotic adjustments in hyponatremic patients are made through a loss of erythrocyte potassium. Although they do not differentiate primary cellular potassium loss and secondary hyponatremia from primary hyponatremia and secondary cellular potassium loss, the present data are in agreement with the findings of others.[14, 15] It should be noted that in hyponatremic subjects the total erythrocyte potassium content was found to be decreased by Agna and Knowles.[16] However, the adjustments involved in achieving osmotic equilibrium are more complex than the simple movement of electrolytes and/or water into and out of the cells,[28, 29] although ultimately this may be expected to occur. Additional factors of importance in the movement of sodium, potassium, and water across the cell membranes include the osmotic effects of glucose and nonprotein nitrogen,[30] pH,[31, 32] the rate of glycolysis,[33] and adrenal cortical activity.[26, 34–36] Since the majority of patients in the study did not have abnormal concentrations of glucose or nonprotein nitrogen in the plasma, and there was no reason to suspect acid-base imbalance, except in those few with renal or respiratory acidosis, it is difficult to ascribe the observed alterations to osmotic or pH changes alone.

On the other hand it is well recognized that aldosterone activity is increased in edema.[37–39] It is tempting to speculate that alterations in aldosterone activity are responsible for the changes in erythrocyte sodium concentration observed in our edematous patients. This thesis can be supported by the following observations. Woodbury and Koch[40] have observed that the administration of aldosterone to mice produces a decrease in muscle sodium concentration. Kumar and co-workers[41] found that in preeclamptic women muscle sodium content was inversely related to aldosterone excretion. Salt deprivation, which causes a rise in aldosterone excretion,[42] has been associated with a fall in the concentration of sodium in the erythrocytes.[43] Aldosterone administered to nephrectomized rats or rats with diabetes insipidus results in an increase in the total extracellular sodium content.[44] Data from our laboratory suggest that erythrocyte sodium concentration declines after the intravenous administration of aldosterone.[45] With respect to the rise in erythrocyte sodium concentration during diuresis, however, the thesis set forth is weakened somewhat by the

recent observation that mercurial diuretics may induce a shift of sodium into the red cells.[46] Therefore, whereas increased aldosterone activity may be responsible in part for the movement of sodium out of the red cells in edematous patients actively retaining fluid, there is no good evidence to indicate that decreased aldosterone actively accounts for the entry of sodium into the erythrocytes during diuresis.

The data in the observations presented provide no clear explanation for the movement of water across the erythrocyte membrane in normonatremic subjects with edema and without acid-base disturbances. Whether the movement is secondary to the electrolyte changes as suggested by Yannet and Darrow,[28] or whether antidiuretic hormone can be implicated as an independent factor cannot be stated.[47, 48]

SUMMARY

1. Fifty-six determinations of erythrocyte electrolyte and water concentration were made in thirty-one edematous patients with renal disease, cirrhosis with ascites, and congestive failure. The results were compared with the findings in twenty-four normal subjects.

2. The data indicate that during the accumulation of edema, or when there is therapeutic unresponsiveness, the concentration of sodium in the red cells is decreased and water increased. Red cell potassium concentration is not changed significantly suggesting an increase in total potassium content. The sodium concentration and water content are the same in both hypo- and normonatremic patients, suggesting that the intracellular migration of sodium does not contribute to hyponatremia in edematous patients, so far as the red cells might reflect changes in other tissue cells. The concentration of potassium, however, is significantly decreased in the hyponatremic patients when compared to the normonatremic group. During diuresis the erythrocyte sodium concentration increases and declines again as diuresis ceases.

3. It was noted that osmotic changes or changes in pH alone did not explain the results obtained. Increased aldosterone activity was suggested as one of the factors influencing movement of sodium out of the red cells in edematous patients, but there was no evidence that decreased aldosterone activity induced the transfer of sodium into the erythrocytes.

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A MICRO MODIFICATION OF A METHOD FOR THE MEASUREMENT OF GLOBULIN IN CEREBROSPINAL FLUID AND ITS APPLICATION IN DIAGNOSIS OF MULTIPLE SCLEROSIS

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A micro modification of a method for the determination of gamma globulin in cerebrospinal fluid is presented together with a micro method for the determination of total protein in the fluid. A single determination of gamma globulin (0.2 ml.) and of total protein (0.1 ml.) requires a total of 0.3 ml. of fluid. The results are correlated with a study of thirteen hospitalized patients with multiple sclerosis and thirty-two patients with nondemyelinating diseases. A significant difference both in gamma globulin concentration and in the ratio of the gamma globulin to the total protein concentration is shown for these two groups.

IN attempting to apply the method reported by Roboz and co-authors[1] for the determination of γ-globulin in spinal fluid to the diagnosis of multiple sclerosis, the problem of inadequate quantities of spinal fluid is often encountered. The reported method[1] requires 2 to 5 ml. of fluid for a single determination as well as an additional unspecified volume for total protein determination.

The present study describes a micro modification of the reported procedure of Roboz and co-workers[1] which requires 200 μl of spinal fluid for the γ-globulin determination and 100 μl of fluid for the total protein determination. These modified procedures have been applied to the determination of γ-globulin and total protein in spinal fluids obtained from patients with either demyelinating or nondemyelinating diseases.

MATERIALS AND METHODS

The Coleman Universal model 14 spectrophotometer with the long light path cuvettes described by Sobel and Snow[2] was employed for all colorimetric readings. The reagents used were those described by Roboz and associates,[1] with minor modifications.

Reagents.—

1. Zinc barbital buffer (pH 7.3; stock). Dissolve 1.45 Gm. of $ZnSO_4 \cdot 7H_2O$, 3.60 Gm. of barbital, and 1.50 Gm. of sodium barbital in 500 ml. of hot water. Cool to room temperature and dilute to 1,000 ml. with water. Adjust pH to 7.3 with sodium hydroxide. Add 1 ml. of chloroform and store in the refrigerator.

2. Zinc alcoholic buffer. Mix 1,000 ml. of the stock buffer with 100 ml. of 95 per cent ethyl alcohol. Store in the refrigerator.

3. Zinc barbital buffer (dilute). Dilute 500 ml. of the stock buffer to 1,000 ml. with water. Add 1 ml. of chloroform and store in the refrigerator.

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4. Cupric sulfate (0.05 per cent). Dissolve 0.5 Gm. of $CuSO_4 \cdot 5H_2O$ in 1,000 ml. of water.

5. Sodium carbonate (12.5 per cent). Dissolve 125 Gm. of anhydrous Na_2CO_3 in water and dilute to 1,000 ml.

6. Sodium carbonate (1.25 per cent). Dilute 100 ml. of the 12.5 per cent solution to 1,000 ml. with water.

7. Phenol reagent (Folin-Ciocalteau). The stock reagent was purchased commercially and had been prepared according to the method of Folin and Ciocalteau.[3] The stock reagent (4 ml.) is diluted with 6.0 ml. water before use.

Procedure.—

Gamma globulin determination: Deliver 200 μl of spinal fluid into the bottom of a 15 ml. glass-stoppered centrifuge tube. Add 200 μl of chilled zinc alcoholic buffer. Tap vigorously and allow to stand for 10 minutes. Mix once more by tapping and place in the refrigerator overnight. Unstopper and centrifuge in chilled cups for 10 minutes at 1,325 g. Decant and discard supernatant fluid. Wash the precipitate twice with 400 μl of chilled dilute zinc barbital buffer, centrifuging each time in chilled cups for 5 minutes at 1,325 g. Tap the tubes vigorously to suspend the precipitate in the wash reagent. Decant and discard the supernatant fluid. After the second wash, invert the tubes over a gauze pad for 10 minutes to drain dry.

Total protein determination: Deliver 100 μl of spinal fluid into the bottom of a 15 ml. glass-stoppered centrifuge tube.

Analysis for γ-globulin and total protein: To all tubes add 500 μl each of 1.25 per cent sodium carbonate and 0.05 per cent cupric sulfate, followed by 2.0 ml. of 12.5 per cent sodium carbonate. Mix thoroughly after each addition. Stopper the tubes and place in a 37° C. incubator for 30 minutes. Remove tubes from incubator and add 1.0 ml. of diluted phenol reagent. Stopper, shake vigorously, and let stand for 10 minutes. Pour into long light path cuvettes and read at 460 mμ against a blank set at zero density. The blank is prepared individually for the γ-globulin determination and the total protein determination and consists of all reagents and steps involved in each determination, using water instead of spinal fluid.

Standardization of Methods.—Fresh, normal human sera were pooled and electrophoretic determinations of the γ-globulin concentration were performed on 10 aliquots. Total protein was determined by the biuret technique on 10 aliquots as well as by duplicate Kjeldahl analysis on a single aliquot. All results were averaged (Table I) and the concentration of γ-globulin was calculated. Dilutions of the pooled serum were made with physiologic saline, giving a series of standards for γ-globulin as well as for total protein. The standards were then treated as described in the previous sections and the optical densities were plotted against concentration. The curves are not linear and should be established with each set of determinations. Standards containing 1.0, 2.0, 4.0, 8.0, and 16.0 mg. per cent γ-globulin and 2.0, 4.0, 8.0, 16.0, and 32.0 mg. per cent total protein were found convenient for providing the calibration curves.

RESULTS AND DISCUSSION

As seen in Table I, the agreement between samples for the determination of total protein concentration (7.2 ± 0.06 Gm. per cent) was quite good as well as the agreement between the biuret and Kjeldahl techniques. The γ-globulin analyses also showed a high degree of precision (1.64 ± 0.07 Gm. per cent).

Evaluation of Methods.—Spinal fluids were analyzed for γ-globulin and for total protein by the methods described. Equal volumes of the various standards used were added to aliquots of these spinal fluids and the γ-globulin

and total protein concentrations of the mixtures determined. The average recovery of γ-globulin was 95.4 per cent and the average recovery of total protein was 104.6 per cent (Table II). The γ-globulin recovery agrees well with that reported by Roboz and co-workers.[1] These authors do not present data for recovery of total protein.

TABLE I. TOTAL PROTEIN AND GAMMA GLOBULIN ANALYSES ON POOLED HUMAN SERA

METHOD	NO. OF DETERMINATIONS	TOTAL PROTEIN (GM. %)	GAMMA GLOBULIN* (GM. %)
Biuret	10	7.2 ± 0.06†	
Kjeldahl	2	7.18	
Electrophoresis	10		1.64 ± 0.07†

*The γ-globulin concentration was determined from the total protein and the per cent γ-globulin found for each aliquot.
†Mean plus or minus standard deviation.

TABLE II. RECOVERY OF TOTAL PROTEIN AND GAMMA GLOBULIN ADDED TO SPINAL FLUID

TOTAL PROTEIN			GAMMA GLOBULIN		
ADDED (MG. %)	FOUND (MG. %)	RECOVERY (%)	ADDED (MG. %)	FOUND (MG. %)	RECOVERY (%)
28.2	31.0	110.0	8.6	8.7	101.0
42.4	45.0	106.0	17.7	14.4	81.5
52.7	52.0	98.5	5.6	5.4	96.5
28.2	26.5	94.0	10.0	8.9	89.0
39.0	41.0	105.0	17.4	16.4	94.5
63.2	64.5	102.0	4.3	4.0	93.0
82.5	97.0	117.0	12.8	14.0	109.0
Average		104.6	17.5	16.0	92.0
			32.8	30.0	92.0
			Average		95.4

TABLE III. AVERAGE VALUES FOR GAMMA GLOBULIN CONCENTRATIONS OF SPINAL FLUIDS*

DIAGNOSIS	GAMMA GLOBULIN (MG. %)	GAMMA GLOBULIN (%)
Multiple sclerosis†	7.6 ± 3.94	23.3 ± 8.15
Nondemyelinating disease‡	3.0 ± 1.05	12.2 ± 3.38

*Values are means plus or minus standard deviations.
†Thirteen patients in the neurosurgical service of the Bronx Municipal Hospital Center.
‡Thirty-two patients.

Application of Methods.—Roboz and associates[1] present data showing that the γ-globulin concentrations of spinal fluid are significantly higher in patients with multiple sclerosis as compared to fluids from patients with nondemyelinating disease. Their data also indicate that the per cent ratio of γ-globulin to total protein in spinal fluid is also significantly higher in the fluid obtained from patients with multiple sclerosis. From review of their data it is apparent that there is some overlapping both in the γ-globulin concentration and the per cent γ-globulin in cases where these values may be as high or higher in nondemyelinating disease than the lowest reported values for multiple sclerosis. It was felt, therefore, that a more rigid criterion of demyelinating disease would be the concomitant elevation of both of these parameters. In order to test this suggestion and to evaluate the methods reported, 45 spinal fluids were chosen at

random; γ-globulins were determined and γ-globulin per cents of the total proteins were calculated. Thirteen of these fluids were from patients with multiple sclerosis and 32 were from patients with some form of nondemyelinating disease. The results of this study are presented in Table III. The mean γ-globulin concentrations in spinal fluids from patients with multiple sclerosis were found to be 7.6 ± 3.94, whereas in fluids from patients with nondemyelinating disease the values were 3.0 ± 1.05. Similarly the per cent γ-globulin figures were, respectively, 23.3 ± 8.15 and 12.2 ± 3.38. The differences between the means were analyzed using the "t" test as described by Youden.[4] The differences were found to be highly significant. In no case was the γ-globulin concentration in the group with multiple sclerosis found to be less than the mean minus one standard deviation and in only three cases was the γ-globulin concentration higher than the mean plus one standard deviation in the group with nondemyelinating disease. In only one of these three cases was the γ-globulin found to be higher than the mean plus two standard deviations. However, in this particular case it may be that the diagnosis of cerebellar degeneration was the prime disease and demyelinating changes may have existed.

In only two cases was the per cent γ-globulin lower in the group with multiple sclerosis than the mean less one standard deviation. In both these cases the γ-globulin was found to be appreciably above the mean for the group. Roboz and collaborators[1] believe that this relationship may indicate a different phase of the disease. In the nondemyelinating group, four patients had per cent γ-globulins that were higher than the mean plus one standard deviation. Again one of these cases was that diagnosed as cerebellar degeneration.

Since there is some overlap of data present in the areas of plus or minus one standard deviation both for the γ-globulin and the per cent γ-globulin in multiple sclerosis and in nondemyelinating diseases, the following criteria are being proposed for the laboratory confirmation of multiple sclerosis: that the γ-globulin as well as the per cent γ-globulin must be elevated; that when the γ-globulin concentration is close to the mean less one standard deviation, the per cent γ-globulin must be well elevated; that when the γ-globulin is elevated with a low per cent γ-globulin (high total protein), the results should not be viewed as conclusive for the diagnosis of multiple sclerosis but the fluid should be examined for the presence of serum proteins.

In addition it is also suggested that all determinations be done in duplicate and the average of the duplicates be reported.

SUMMARY

1. A method has been presented for the determination of γ-globulin in small volumes of spinal fluid. This method is a modification of a method in the literature.

2. In addition, a method has been presented for the determination of total protein in small volumes of spinal fluid.

3. These methods have been applied to the study of spinal fluids from patients with multiple sclerosis.

4. The observation has been made that both γ-globulin concentration and per cent γ-globulin of the total protein found are significantly elevated in those spinal fluids from patients with multiple sclerosis.

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EFFECT OF ESTROGEN ADMINISTRATION ON HYDROCORTISONE METABOLISM IN MAN

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Ethinyl estradiol, administered orally in the daily dosage of 1 mg., produced a prompt increase of free plasma hydrocortisone levels in 16 men of varying ages with and without liver disease. Despite elevated free plasma 17-hydroxycorticosteroids, conjugated plasma and urinary 17-hydroxycorticosteroid values did not rise and actually fell slightly in several patients during estrogen therapy. The free plasma corticosteroid clearance in response to a standard infusion of hydrocortisone was impaired in patients who had received estrogen as was the formation of plasma 17-hydroxycorticosteroid conjugates. The effects of ethinyl estradiol on hydrocortisone metabolism may be due to an increase in plasma protein binding of hydrocortisone and also to a decrease in the capacity of the liver to reduce and conjugate hydrocortisone.

VARIOUS investigators have demonstrated that the oral administration of estrogens to man results in elevated levels of free plasma 17-hydroxycorticosteroids.[1, 2] Despite these high plasma values of hydrocortisone, patients who have received estrogen therapy for long periods of time do not develop evidence of hypercorticism.[2, 3] In fact, the administration of diethylstilbestrol to a female patient with Cushing's syndrome has been reported as resulting in a clinical remission of the disease.[4] In order to explain what seems at first glance to be a physiologic paradox, we should like to describe our observations concerning the effects of ethinyl estradiol on hydrocortisone metabolism in men.

MATERIALS

Sixteen hospitalized men varying in age from 21 to 69 years were selected for study. For the most part these patients were in a good state of nutrition and were convalescing from an orthopedic disease. Four patients had clinical and laboratory evidence of liver disease. Each patient received 1 mg. of ethinyl estradiol orally in a divided dosage of 0.5 mg. twice a day continuously for varying intervals up to 14 days. In order to test adrenocortical capacity, 30 international units of ACTH was given intravenously over a 6 hour period and the free plasma 17-hydroxycorticosteroid response was determined. Plasma hydrocortisone clearances were studied by infusing hydrocortisone (1 mg. per kilogram) over a 30 minute period and then measuring the free and conjugated plasma 17-hydroxycorticosteroid levels for 6 hours after the infusion.

Plasma free 17-hydroxycorticosteroids (17-OHCS) were determined by the method of Nelson and Samuels.[5] Conjugated plasma 17-hydroxycorticosteroids were analyzed by a

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modification of the method of Bongiovanni and Eberlein,[6] free and conjugated urinary 17-hydroxycorticosteroids by a modification of the method of Glenn and Nelson.[7]

RESULTS

The plasma values of free and of conjugated plasma 17-hydroxycorticosteroids both before and after the administration of ethinyl estradiol for a period of 1 to 14 days are shown in Table I. There was a uniform increase in free plasma 17-hydroxycorticosteroid levels in patients of all ages regardless of the presence or absence of liver disease. The elevation of free plasma 17-hydroxycorticosteroids occurred as promptly as 48 hours after the onset of estrogen therapy. It is of interest that the conjugated plasma 17-hydroxycorticosteroid values not only did not rise but actually fell slightly in many patients during estrogen administration.

The minimal increase in free plasma corticoid levels in patients F. E. and F. R. may be related to the fact that these individuals had been on prolonged triamcinolone therapy prior to the study. Both patients had evidence of corticosteroid induced adrenocortical suppression, which was demonstrated by a diminished plasma 17-hydroxycorticosteroid response to intravenous ACTH. A constant daily dose of 6 mg. of triamcinolone was continued throughout the period of ethinyl estradiol administration in these patients.

In Table II are recorded the free and the conjugated urinary 17-hydroxycorticosteroid values of seven patients both before and after 2 weeks of ethinyl estradiol therapy. There was a small decrease in the conjugated glucuronide fraction in five patients after estrogen therapy; free urinary 17-hydroxycorticosteroid excretion showed a minimal increase in all patients. The effects of ethinyl estradiol on both the free and the conjugated plasma and urinary 17-hydroxycorticosteroid levels of patient G. R. are in Fig. 1.

TABLE I. EFFECT OF ETHINYL ERTRADIOL ON FREE AND CONJUGATED PLASMA 17-OHCS LEVELS

PATIENT	AGE (YR.)	PRE-ETHINYL ESTRADIOL PLASMA 17-OHCS		DAYS OF THERAPY	POSTETHINYL ESTRADIOL PLASMA 17-OHCS	
		FREE (γ/100 ML.)	CONJUGATES (γ/100 ML.)		FREE (γ/100 ML.)	CONJUGATES (γ/100 ML.)
M. A.	21	21	17	14	56	15
W. E.	28	18	22	14	64	19
T. S.	32	17	14	2	27	16
K. I.	37	19	13	3	53	14
R. J.	40	20	15	3	39	13
G. R.	45	21	22	14	46	18
A. B.	53	16	20	14	59	13
T. M.	56	12	18	14	42	14
F. E.	62	6	9	10	15	10
F. R.	64	9	11	14	21	8
M. N.	67	22	15	14	49	16
R. E.	69	18	12	14	50	15
Patients With Liver Disease.—						
P. O.	27	14	15	14	55	12
D. U.	37	19	12	14	46	10
D. R.	62	13	16	14	41	17
F. A.	63	13	19	14	53	13

TABLE II. EFFECT OF TWO WEEKS OF ETHINYL ESTRADIOL ADMINISTRATION ON THE RENAL EXCRETION OF FREE AND CONJUGATED 17-OHCS

	PRE-ETHINYL ESTRADIOL		POSTETHINYL ESTRADIOL	
PATIENT	FREE (MG./24 HR.)	CONJUGATES (MG./24 HR.)	FREE (MG./24 HR.)	CONJUGATES (MG./24 HR.)
M. A.	0.29	5.2	0.43	4.8
W. E.	0.21	4.4	0.36	3.5
G. R.	0.10	3.5	0.18	2.9
A. B.	0.32	6.1	0.57	4.8
T. M.	0.25	5.8	0.38	5.1
M. N.	0.18	4.2	0.29	4.1
R. E.	0.45	4.7	0.51	5.2

The same patient was given a 6 hour ACTH infusion both before and after ethinyl estradiol administration. The results are illustrated in Fig. 2. The free plasma 17-hydroxycorticosteroid response prior to estrogen therapy was within the normal range, i.e., from 21 to 46 γ per cent. After 2 weeks of ethinyl estradiol therapy, a markedly exaggerated free plasma corticoid response to ACTH was observed, i.e., from 46 to 122 γ per cent. In spite of this exaggerated free plasma steroid response to ACTH, the conjugated urinary 17-hydroxycorticosteroid response to ACTH was slightly less after estrogen therapy, i.e., 11.9 mg. per 24 hours before estrogen as compared to 10.8 mg. per 24 hours after estrogen. The urinary free 17-hydroxycorticosteroid response to ACTH was minimally increased after estrogen. Similar results were observed in four patients.

In Fig. 3, the free and conjugated plasma 17-hydroxycorticosteroid levels, expressed logarithmically, of patient G. R. after a standard infusion of hydrocortisone both before and after estrogen therapy are plotted against time. It may be seen that after ethinyl estradiol administration for 2 weeks, much higher free plasma 17-hydroxycorticosteroid values were reached, the disappearance of free hydrocortisone from plasma was markedly delayed, and the formation of conjugated plasma 17-hydroxycorticosteroids was impaired in response to the hydrocortisone infusion. Similar results of the effect of estrogen therapy on free plasma hydrocortisone clearance were observed in five other patients and are recorded in Table III.

During ethinyl estradiol administration, patients F. E. and F. R. exhibited clinical phenomena worthy of comment. Patient F. E. suffered from chronic neurodermatitis and patient F. R. had rheumatoid arthritis of several years'

TABLE III. EFFECT OF ETHINYL ESTRADIOL ON FREE PLASMA 17-OHCS LEVELS AFTER A STANDARD INFUSION OF HYDROCORTISONE

		PRE-ETHINYL ESTRADIOL					POST-ETHINYL ESTRADIOL			
PATIENT	CONTROL	1 HR. (γ/100 ML.)	2 HR. (γ/100 ML.)	4 HR. (γ/100 ML.)	6 HR. (γ/100 ML.)	CONTROL	1 HR. (γ/100 ML.)	2 HR. (γ/100 ML.)	4 HR. (γ/100 ML.)	6 HR. (γ/100 ML.)
M. A.	21	81	56	28	15	56	176	138	108	97
W. E.	18	96	63	32	16	64	154	150	113	78
A. B.	16	87	59	31	17	59	169	146	122	83
T. M.	12	75	51	24	11	42	135	105	83	54
P. O.*	14	99	73	49	32	55	163	117	99	81

*Patient P. O. had liver disease and a 45 minute sulfobromophthalein retention of 31 per cent.

duration. Each patient had been maintained in clinical remission for 2 to 3 months prior to our study on constant daily doses of triamcinolone. Within a period of 10 to 14 days after the onset of estrogen therapy, both patients experienced a mild clinical exacerbation of their diseases. Ethinyl estradiol was stopped and triamcinolone was continued in the same daily dosage, i.e., 6 mg. These clinical exacerbations then were observed to gradually subside over a period of 2 to 3 weeks in both patients.

DISCUSSION

It appears from this study that the administration of ethinyl estradiol to men produces marked alterations in the metabolism of hydrocortisone. Our results are similar to those reported by Migeon and associates,[8] concerning the physiologic disposition of radioactive hydrocortisone during late pregnancy. Other investigators have confirmed Migeon's observations concerning the effects of pregnancy on adrenal steroid metabolism.[9, 10] The delayed disappearance of infused hydrocortisone from the plasma and the exaggerated free plasma 17-hydroxycorticosteroid response to intravenous ACTH after estrogen therapy have been documented by Wallace and Robertson and associates.[2, 11]

In order to explain these phenomena, it is necessary to consider the concept of plasma protein binding of hydrocortisone.[12, 13] If minute quantities of radioactive hydrocortisone are added to dilute human plasma and this plasma is then dialyzed against a solution of one per cent human serum albumin until equilibrium is reached, approximately 85 to 90 per cent of the radioactivity remains with the plasma apparently bound to the alpha globulin fraction. [12, 13]

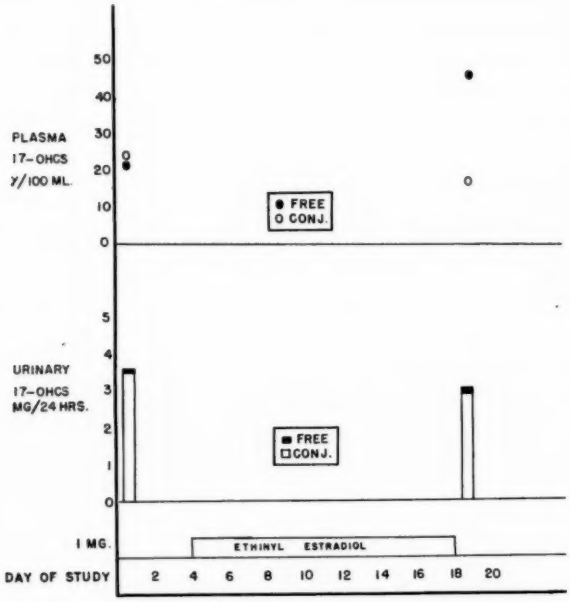

Fig. 1.—The effect of 2 weeks of ethinyl estradiol administration on the free and the conjugated plasma and urinary 17-OHCS values of patient G. R., a 45-year-old man.

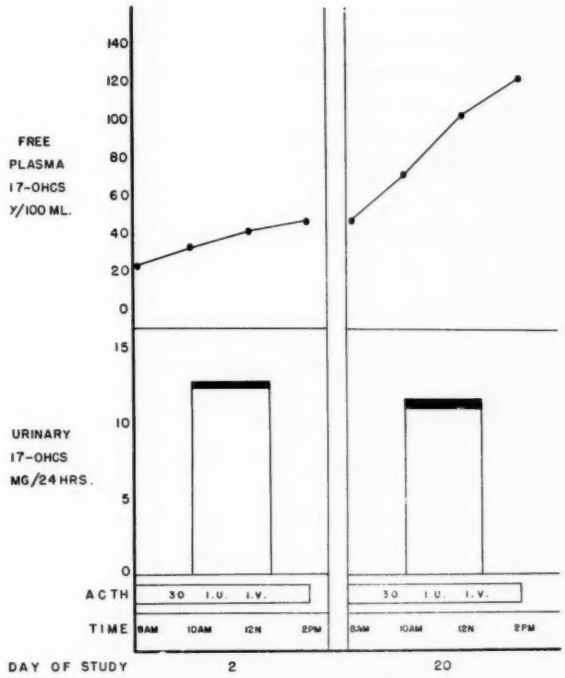

Fig. 2.—The effect of estrogen therapy on the response of free plasma 17-OHCS levels and free and conjugated urinary 17-OHCS excretion to 30 I.U. of intravenous ACTH in patient G. R. Ethinyl estradiol was given daily (0.5 mg. twice a day) from day 4 to day 18 of the study.

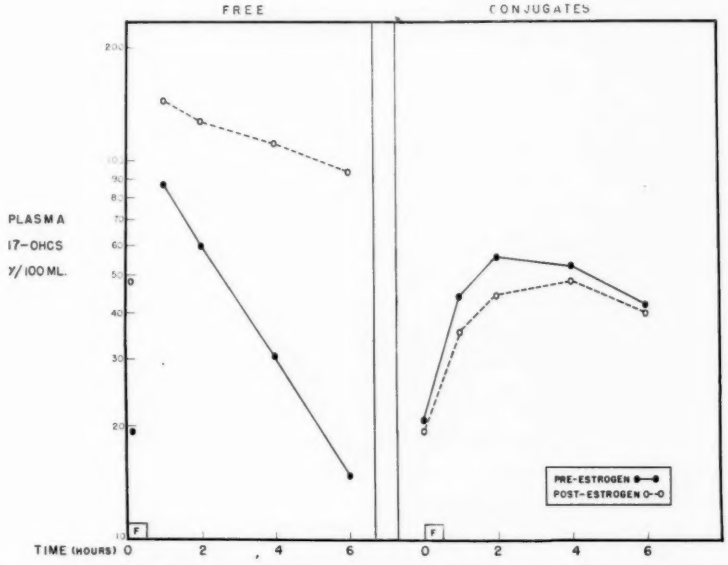

Fig. 3.—The effect of 2 weeks of ethinyl estradiol therapy on the free and conjugated 17-OHCS levels after the infusion of hydrocortisone (1 mg. per kilogram) to patient G. R.

This corticosteroid-binding protein in plasma has been called transcortin by Slaunwhite and Sandberg[14] and has been demonstrated to bind hydrocortisone 6,000 times more strongly than does albumin. The affinity of transcortin is quite specific and conjugated metabolites of hydrocortisone are bound very weakly, if at all, by transcortin.[3] The transcortin capacity of human plasma has been shown to be greatly increased during pregnancy and especially during the administration of estrogens.[3, 14] The effects of estrogens on the transcortin levels occur within a few days after their administration and the transcortin capacity progressively rises with continuation of the therapy.[3] Since the increase in free plasma hydrocortisone values both during pregnancy and after estrogen therapy is not accompanied by the classic signs of hypercorticism, it has been suggested that hydrocortisone bound to transcortin is biologically inactive.[11, 14]

Mills and Bartter[15] have suggested that the elevated free plasma 17-hydroxycorticosteroid levels observed during estrogen therapy are related primarily to an increase in the protein bound hydrocortisone fraction. It seems logical to assume that plasma protein binding of hydrocortisone protects the circulating hormone to some extent from catabolism by the liver. This concept could explain the decreased rate of removal of infused hydrocortisone during estrogen therapy, but it does not satisfactorily account for the following observations: (1) during ethinyl estradiol administration to adult patients, conjugated urinary 17-hydroxycorticosteroid excretion falls, but free urinary 17-hydroxycorticosteroid values remain unchanged or increase slightly; (2) the plasma clearance of tetrahydrocortisone has been shown to be slower in pregnant than in normal women.[10]

These phenomena can be explained by the thesis that increased circulating levels of estrogen either in normal men or pregnant women may diminish the rate of hepatic metabolism of hydrocortisone. Troop[16] showed that the daily injection of estradiol-17β to gonadectomized adult rats decreased the capacity of rat liver to reduce the 17,21-dihydroxy-20-ketone side chain of cortisone.

The effects of ethinyl estradiol on plasma 17-hydroxycorticosteroid conjugates and on conjugated urinary 17-hydroxycorticosteroid excretion are worthy of comment, for a decrease in these values occurred in many of our patients. Peterson[17] measured conjugated urinary 17-hydroxycorticosteroid excretion and also the turnover rate of radioactive hydrocortisone both before and after 4 weeks of ethinyl estradiol therapy in two patients and found that these sensitive indices of adrenocortical function decreased by 40 per cent. These observations are consistent with the concept that ethinyl estradiol, in the dose used in this study, is capable of depressing the adrenal secretion of hydrocortisone in man.

In light of the foregoing discussion, it seems appropriate to offer a theoretical explanation for the mild clinical exacerbations observed in patients F. E. and F. R., during ethinyl estradiol administration. A diminished free plasma 17-hydroxycorticosteroid response to intravenous ACTH was demonstrated in each patient prior to estrogen therapy and indicated the presence of corticosteroid induced adrenocortical suppression. It is suggested that these individuals had a relatively fixed daily supply of corticosteroids in the form of a

constant dose of triamcinolone plus a small amount of endogenous hydrocortisone secreted by partially suppressed adrenal glands. This total daily supply of adrenal steriods may have sufficed to maintain a clinical remission in these patients prior to estrogen therapy. The administration of ethinyl estradiol could have produced a slight decrease in the quantity of biologically active adrenal hormone available to the diseased tissues by depressing further the already diminished adrenal secretion of hydrocortisone and/or by increasing the plasma protein corticosteroid-binding capacity, which might have resulted in an increased retention of hydrocortisone within the intravascular compartment.

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A METHOD FOR DEMONSTRATING PROBABLE SYNTHESIS OF PROTEIN BY BONE MARROW CELLS

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This study was undertaken in an attempt to demonstrate in vitro synthesis of protein by bone marrow cells in multiple myeloma and other paraproteinemias. Bone marrow aspirates and cell-free plasma were incubated with tritiated DL-leucine, dialyzed, and electrophoretically separated. The paper electrophoretic strips were then assayed for incorporation and localization of radioactivity. Autoradiographs of marrow cells were made utilizing a liquid emulsion technique. In 3 patients with multiple myeloma, it was possible to demonstrate uptake of radioactivity by myeloma cells and its incorporation into myeloma protein. Radioactivity was also observed in lymphoid reticulum cells and the abnormal protein in a patient with Waldenström's macroglobulinemia. Incorporation of radioactivity into the globulin fraction was demonstrated in a patient with idiopathic hypergammaglobulinemia associated with carcinomatosis and in a patient with cirrhosis. Normal marrow and the cell-free plasma from all patients failed to demonstrate significant incorporation of radioactivity into any protein fraction. The method presented appears to demonstrate synthesis of protein of several types, normal and abnormal, and serves to identify the cells most likely involved in such synthesis.

FOR many years it has been suggested that the reticuloendothelial tissues of the body are intimately involved in the synthesis of gammaglobulin. One of the most striking examples of this relationship is the frequent occurrence of an increase of an electrophoretically homogeneous plasma globulin associated with multiple myeloma. Three types of evidence have been presented which suggest a causal relationship between the myeloma protein and the abnormal plasma cell in the bone marrow in this disease: (1) with destruction or extirpation of a myeloma tumor, or with reduction in the marrow plasmocytosis in response to urethane, there may be a decrease in the amount of myeloma protein in the plasma[1-3]; (2) extracts of myeloma tissue, tumor, or marrow have yielded a protein which has the characteristics of the serum myeloma protein on the basis of amino acid composition,[4] sedimentation constants,[5] paper electrophoretic patterns,[6] and immunocytochemical studies using fluorescent antibody techniques[7]; and (3) it has been demonstrated that a mouse myeloma tumor transplanted into normal mice produced myeloma protein which appeared in the plasma.[8, 9]

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While these data suggest protein synthesis by myeloma tissue, alternative explanations are possible: (1) the decline in myeloma protein associated with a decrease in myeloma tissue could be explained by postulating the removal of a stimulus for protein synthesis by another organ, such as the liver; (2) the concomitant presence of apparently identical proteins in extracts of myeloma tissue and the plasma could be due to incomplete removal of the plasma from the tissue; (3) it is possible that the protein is synthesized elsewhere in the body and subsequently stored in myeloma tissue. The same objection of possible extratumoral synthesis of protein might be applied to the mouse myeloma tumor, although it seems unlikely that this specific protein could be formed both by the neoplastic cells and by other tissues of the host.[9]

Proof of protein synthesis may be possible by the use of an isolated system independent of the organism, in which production of protein by abnormal cells might be detected. In multiple myeloma the bone marrow, which may be

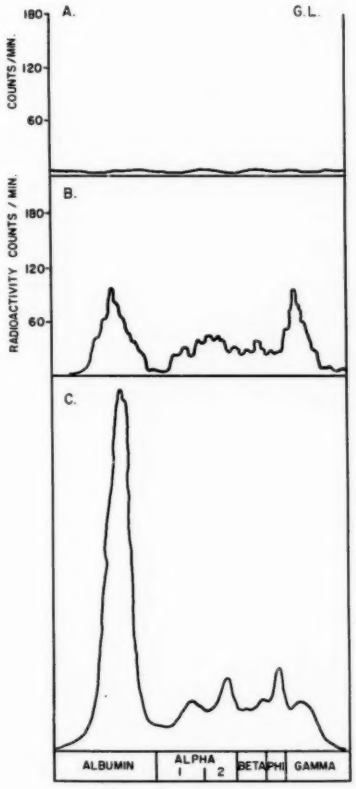

Fig. 1.

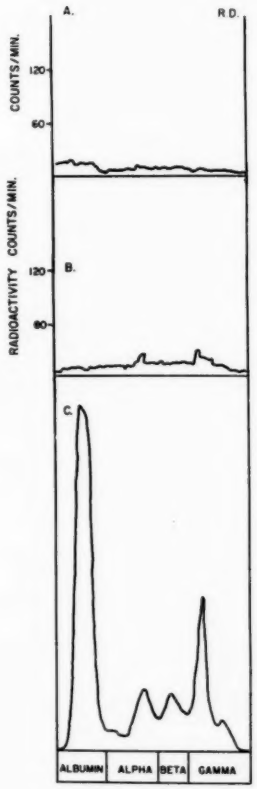

Fig. 2.

Fig. 1.—Patient G. L., normal marrow incubated with H_3 DL-leucine. *A*, Radioscan of electrophoretic strip of marrow plasma after 96 hours' dialysis against distilled water at 20° C. *B*, Same after 48 hours' dialysis. *C*, Densitometer record of marrow plasma electrophoresis.

Fig. 2.—Patient R. D., normal marrow and cell-free plasma incubated with H_3 DL-leucine. *A*, Radioscan of electrophoretic strip of cell-free plasma after 48 hours' dialysis against saline at 4° C. *B*, Radioscan of electrophoretic strip of marrow plasma after 48 hours' dialysis against saline at 4° C. *C*, Densitometer record of marrow plasma electrophoresis.

composed almost entirely of myeloma cells, provides such a system. The present study was undertaken in an attempt to demonstrate myeloma protein biosynthesis by the use of an in vitro method employing tritiated DL-leucine as a protein precursor and to identify the cells involved by the use of autoradiography.

METHODS

Ten milliliters each of bone marrow aspirate and of venous blood were obtained from the eight patients studied. The marrow was aspirated from the iliac crest under local anesthesia with a 14 gauge sternal marrow needle. The marrow and venous blood samples were drawn into heparinized syringes and transferred immediately into heparinized containers. Nucleated cell counts of the marrow aspirate were performed. These ranged from 200 million to 600 million cells per 10 ml. Marrow particles were removed by centrifugation and suspended in 1 to 2 ml. of autologous plasma. Fifty to 100 μc of tritium-labeled DL-leucine was added to 1 to 2 ml. of both the marrow suspension and the plasma. These were incubated for 4 to 20 hours at 37.5° C. After incubation, the marrow suspension was centrifuged and the cell-free plasma was removed.

Plasma from the marrow and from venous blood incubates were dialyzed against distilled water for 24 to 96 hours to remove free leucine. The samples were lyophilized to dryness and redissolved in small amounts of 0.9 per cent saline. Other samples were dialyzed against 0.9 per cent saline at 4° C. for 24 hours but not lyophilized. The latter method proved more satisfactory since shorter periods of dialysis were required to remove the free leucine. Routine paper electrophoresis of each plasma sample was performed using 0.075 molar barbiturate buffer at pH 8.6 in Spinco R cells. Quantitation of the protein on bromphenol blue stained strips was accomplished using a Spinco Analytrol model R. The electrophoretic strips were scanned for radioactivity using a chromatographic scanner with an open-end Geiger-Müller tube and gas flow chamber and utilizing a direct writing recorder. The electrophoretic strips were matched against the recording strip for identification of each protein fraction.

Autoradiographs were prepared in the following manner: the marrow particles were washed three times with warm 0.9 per cent saline and cover slip films of the cells were prepared. These were fixed in absolute methyl alcohol, overstained with Kingsley's stain, dried, and mounted on glass slides. They were then dipped into a 1 per cent solution of celloidin, air dried, and then dipped into a warmed (38°) Kodak NTB-2 liquid photographic emulsion.* The slides were developed after storage in the dark at 4° C. and zero per cent humidity for 10 to 14 days. These autoradiographs were compared with the usual stained marrow preparations for identification of labeled cells.

In view of the limited number of bone marrow cells present and the fact that protein synthesis would be scanty in such a system, an amino acid labeled with high specific activity was required. Tritium-labeled DL-leucine best served this need because of its remarkably high activity of 3,570 mc. per millimole. Suitable controls to exclude nonspecific radioactive tagging of plasma proteins by tritium were required. Accordingly, the plasma derived from each marrow incubation was run in parallel with cell-free plasma incubated with the same quantity of H_2 DL-leucine for similar periods of time. Normal marrow was studied similarly.

RESULTS

1. *Normal Marrow.*—Fig. 1 illustrates the effect of incubation of normal marrow with tritium-labeled leucine after dialysis of plasma against distilled water. Radioactivity was noted in both albumin and globulin fractions after 48 hours' dialysis. After further dialysis for 48 hours, however, all radioactivity was

*The NTB-2 emulsion was supplied by Mr. W. F. Swann of Eastman Kodak Company, Rochester, N. Y.

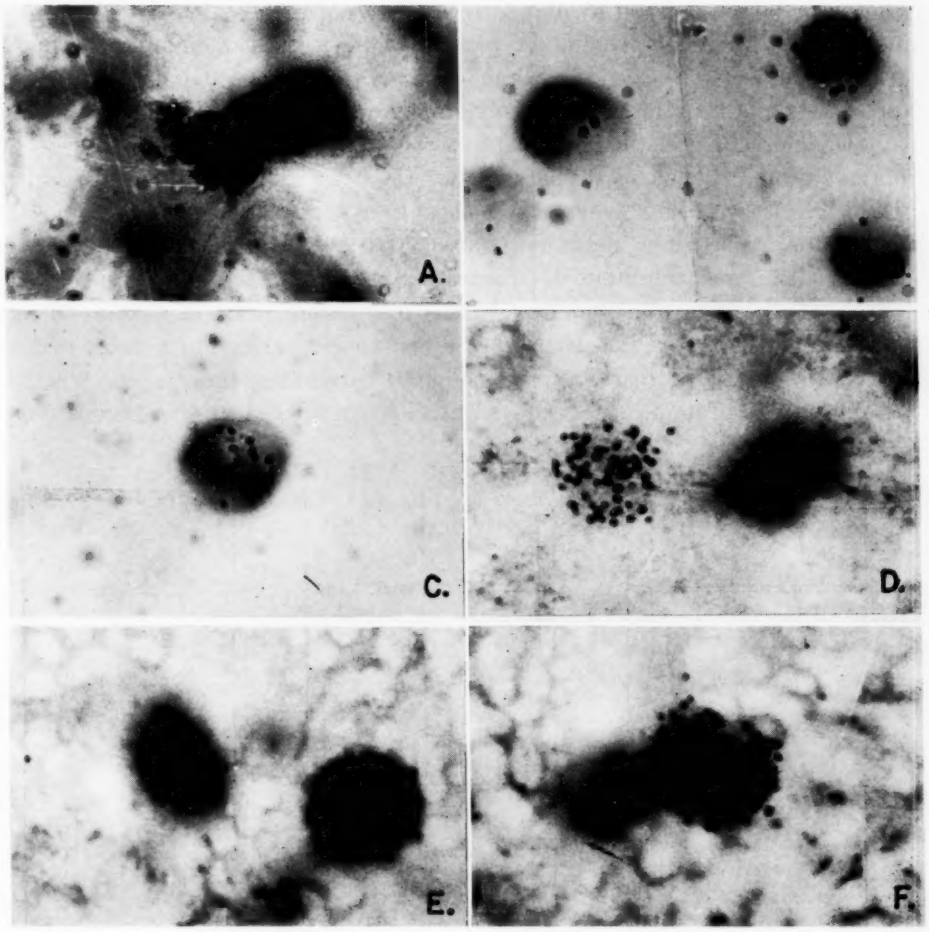

Fig. 3.—Autoradiographs of marrow cells after incubation with H_3 DL-leucine. *A*, Normal marrow; plasma cell. *B*, Multiple myeloma; Patient S. R.; myeloma cells. *C*, Multiple myeloma; Patient S. R.; myeloma cells. *D*, *E*, and *F*, Macroglobulin of Waldenström; lymphoid reticulum cell.

removed. Fig. 2 illustrates the results of similar incubation of another normal marrow sample after dialysis against 0.9 per cent saline at 4° C. With only 24 hours dialysis, all radioactivity was removed. Dialysis against water produced protein precipitates which were difficult to wash free of radioactivity. On the other hand, with dialysis against saline in the cold, the proteins remained in solution and the radioactivity was easily removed.

In neither instance was residual radioactivity noted in the electrophoretic strip. In the autoradiographs of normal marrow no radioactivity was located in any of the marrow cells except in a rare plasma cell (Fig. 3, *A*).

2. *Multiple Myeloma Marrow.*—Marrow samples from three patients with multiple myeloma were studied. Each of these patients presented the classic findings of the disease with bone lesions, marrow replacement by abnormal

plasma cells, and abnormal serum eletrophoretic patterns indicative of paraproteinemia. Two distinct protein components were demonstrated in the urine of one patient (S. R.). Figs. 4, 5, and 6 illustrate the electrophoretic and the radioactivity patterns of each patient's marrow plasma after incubation with H_3 DL-leucine. In each instance a peak of radioactivity corresponding to the abnormal protein bands in the electrophoretic strip was apparent. In patient S. R. (Fig. 4) the radioactive area was sharply defined in the gamma region and demonstrated a threefold increase in radioactivity over the plasma of the other patients with myeloma studied. Repeated studies were performed on this patient. If dialysis was not performed on the plasma prior to electrophoresis, an additional peak of radioactivity was noted corresponding to a faint protein band on the electrophoretic strip immediately preceding the gamma fraction. This dialyzable protein has been tentatively considered to be Bence Jones

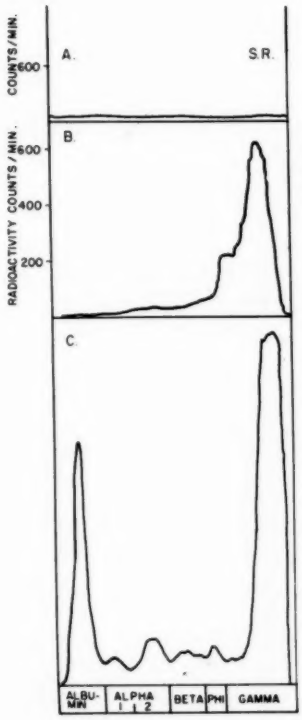

Fig. 4.

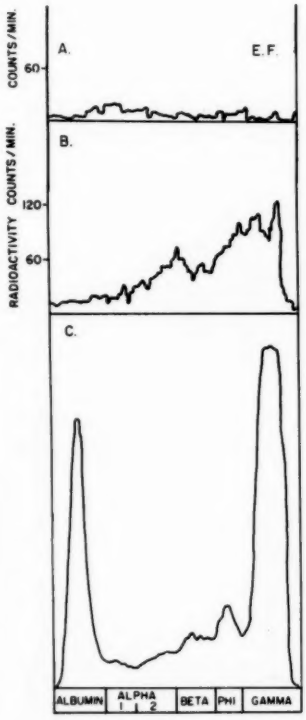

Fig. 5.

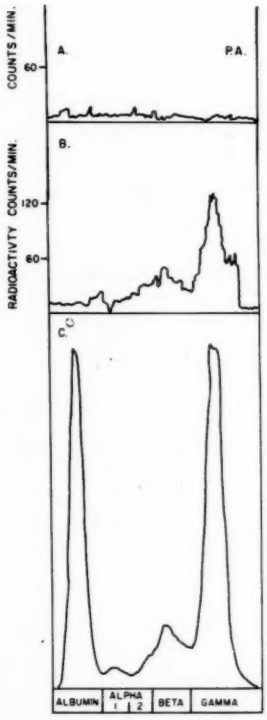

Fig. 6.

Fig. 4.—Patient S. R., multiple myeloma marrow and cell-free plasma incubated with H_3 DL-leucine. *A*, Radioscan of electrophoretic strips of cell-free plasma after 48 hours' dialysis against saline at 4° C. *B*, Radioscan of electrophoretic strip of marrow plasma after 48 hours' dialysis against saline at 4° C. *C*, Densitometer record of marrow plasma electrophoresis.

Fig. 5.—Patient P. A., multiple myeloma marrow and cell-free plasma incubated with H_3 DL-leucine. *A*, Radioscan of electrophoretic strip of cell-free plasma after 48 hours' dialysis against saline at 4° C. *B*, Radioscan of electrophoretic strip of marrow plasma after 48 hours' dialysis against saline at 4° C. *C*, Densitometer record of marrow plasma electrophoresis.

Fig. 6.—Patient E. F., multiple myeloma marrow and cell-free plasma incubated with H_3 DL-leucine. *A*, Radioscan of electrophoretic strip of cell-free plasma after 48 hours' dialysis against saline at 4° C. *B*, Radioscan of electrophoretic strip of marrow plasma after 48 hours' dialysis against saline at 4° C. *C*, Densitometer record of marrow plasma electrophoresis.

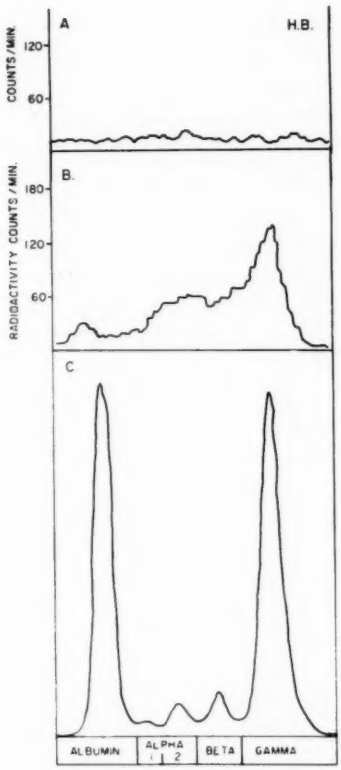

Fig. 7.

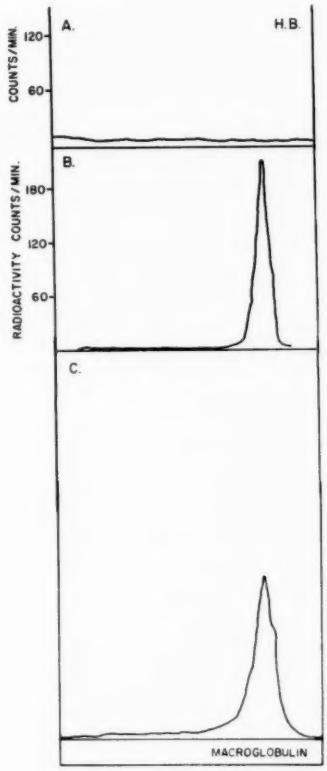

Fig. 8.

Fig. 7.—Patient H. B., macroglobulin of Waldenström marrow and cell-free plasma incubated with H_3 DL-leucine. Pretreatment. *A*, Radioscan of electrophoretic strip of cell-free plasma after 48 hours' dialysis against saline at 4° C. *B*, Radioscan of electrophoretic strip of marrow plasma after 48 hours' dialysis against saline at 4° C. *C*, Densitometer record of marrow plasma electrophoresis.

Fig. 8.—Patient H. B., macrocryogelglobulin isolated by electrolyte-free water precipitation from marrow plasma and cell-free plasma incubated with H_3 DL-leucine. *A*, Radioscan of macroglobulin isolated from cell-free plasma. *B*, Radioscan of macroglobulin isolated from marrow plasma. *C*, Densitometer record of macroglobulin isolated from marrow plasma.

protein. In patients E. F. and P. A. (Figs. 5 and 6) distinct areas of radioactivity corresponding to beta globulin components were present in addition to the major radioactivity in the gamma region. In no instance did the cell-free venous plasma of these patients incubated similarly with H_3 DL-leucine show detectable radioactivity in any protein band.

Autoradiographs of the marrow cells in multiple myeloma were difficult to interpret because of the large amounts of radioactively labeled protein producing a heavy background. Fig. 3, *B* and *C*, demonstrates, however, that in thin smears the radioactivity was usually confined to the area of the cells and overlying them.

3. *Bone Marrow of Macroglobulinemia of Waldenström.*—In patient H. B., a macroglobulin with the ultracentrifugal characteristics of 19 S_{20} Svedberg units which constituted 38 per cent of the G (globulin) fraction was identified. Bone marrow examination demonstrated infiltration by mature lymphocytes,

large lymphoid or plasma reticulum cells, and plasma cells. The macroglobulin possessed characteristics of a macrocryogelglobulin[10] and could be precipitated with 95 per cent purity by the addition of 1.0 ml. of serum to 10 ml. of triple distilled electrolyte free water. Repeated incubation studies were carried out on this patient's marrow before and after treatment with orally administered penicillamine (DL-dimethylcysteine). Fig. 7 depicts the electrophoretic strip and its radioactive scan after incubation with H_3 DL-leucine and dialysis against distilled water before treatment with penicillamine. A well-defined peak of radioactivity was seen in the area of the homogeneous globulin increase. Another sample of the marrow plasma after incubation with H_3 DL-leucine was treated to obtain the pure macroglobulin. One milliliter of the plasma was added to

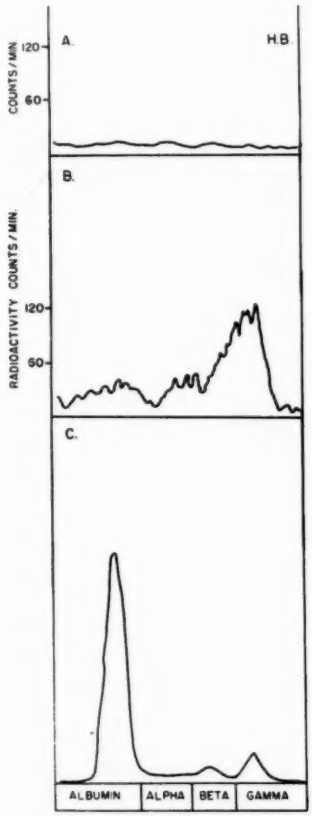

Fig. 9.

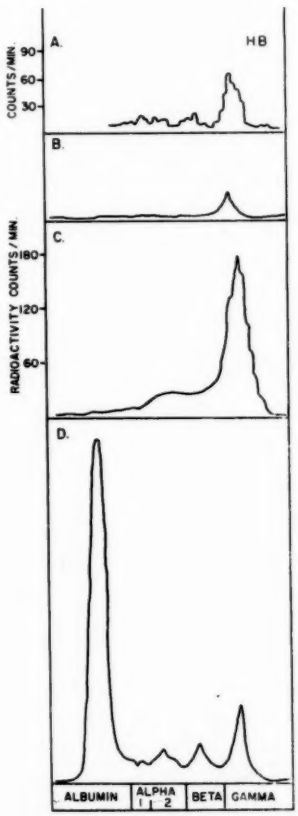

Fig. 10.

Fig. 9.—Patient H. B., macroglobulinemia of Waldenström marrow and cell-free plasma after incubation with H_3 DL-leucine after therapy. *A*, Radioscan of electrophoretic strip of cell-free plasma after 48 hours' dialysis against saline at 4° C. *B*, Radioscan of electrophoretic strip of marrow plasma after 48 hours' dialysis against saline at 4° C. *C*, Densitometer record of marrow plasma electrophoresis.

Fig. 10.—Patient H. B., macroglobulinemia of Waldenström marrow incubated with H_3 DL-leucine. *A*, Radioscan of electrophoretic strip of macroglobulin isolated by electrolyte-free water precipitation and redissolved in 0.9 per cent saline. *B*, Analytrol record of the electrophoretic strip of the isolated macroglobulins. *C*, Radioscan of electrophoretic strip of the supernatant obtained after precipitation of the macroglobulin. *D*, Densitometer record of the electrophoretic strip of the supernatant obtained after precipitation of the macroglobulin and dialyzed against saline for 48 hours at 4° C.

10 ml. of triple distilled electrolyte-free water. The resulting precipitate was washed several times with triple distilled water, dissolved in saline, and separated by paper electrophoresis. A well-defined protein band which demonstrated radioactivity was apparent (Fig. 8). The patient's venous plasma incubated and treated similarly failed to show any radioactivity. Fig. 9 illustrates the

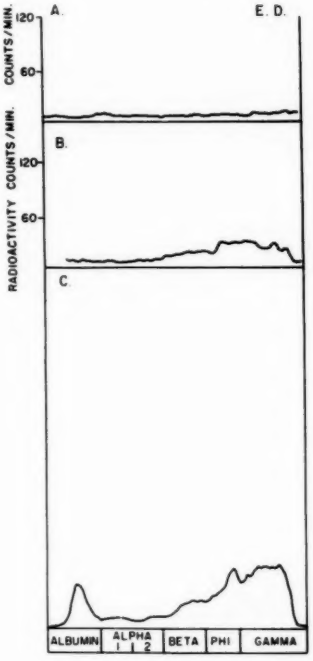

Fig. 11.

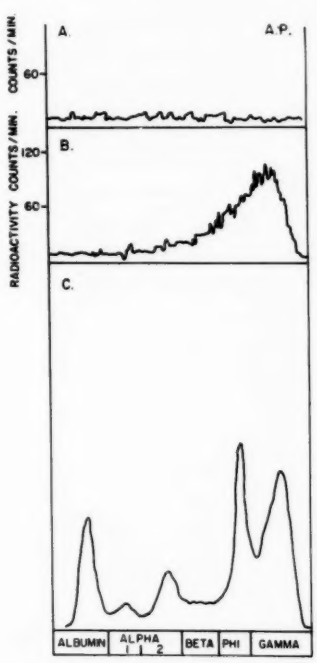

Fig. 12.

Fig. 11.—Patient E. D., cirrhosis of the liver with hypergammaglobulinemia. Marrow and cell-free plasma incubated with H_3 DL-leucine. *A*, Radioscan of electrophoretic strip of cell-free plasma dialyzed against saline at 4° C. *B*, Radioscan of electrophoretic strip of marrow plasma after dialysis against saline at 4° C. *C*, Densitometer record of marrow plasma electrophoresis.

Fig. 12.—Patient A. P., carcinomatosis with hypergammaglobulinemia. Marrow and cell-free plasma incubated with H_3 DL-leucine. *A*, Radioscan of electrophoretic strip of cell-free plasma dialyzed against saline at 4° C. *B*, Radioscan of electrophoretic strip of marrow plasma dialyzed against saline at 4° C. *C*, Densitometer record of marrow plasma electrophoresis.

electrophoretic pattern and radioactivity scan after 3 weeks of penicillamine therapy of the patient. There was a marked reduction in the quantity of the abnormal protein present. As before, after incubation with H_3 DL-leucine, the macroglobulin was precipitated, washed, redissolved in saline, and subjected to electrophoretic separation. The supernatant, after precipitation of the macroglobulin, was dialyzed against distilled water, concentrated by lyophilization, and electrophoresis was performed. Although radioactivity was detected in the purified macroglobulin component, a greater amount was present in the proteins in the supernatant (Fig. 10). Autoradiographs of the marrow cells after H_3 DL-leucine incubation revealed numerous cells possessing radioactivity (Fig. 3, *D*, *E*, and *F*). These were identified as plasma or lymphoid reticulum cells

characteristic of macroglobulinemia,[11] whereas others appeared to be immature plasma cells. Thorough search of the slides failed to disclose any other cell type which contained radioactivity.

4. *Marrow Associated With Miscellaneous Hypergammaglobulinemias.*—Patient E. D., a 65-year-old Negro man with cirrhosis of the liver, demonstrated a broad heterogeneous type of gammaglobulin increase. Fig. 11 indicates the result of H_3 DL-leucine incubation studies. A broad but low area of radioactivity corresponding to the gammaglobulin band was present. Again as in all patients studied, cell-free venous plasma incubated in identical fashion failed to show radioactivity. The bone marrow revealed moderate plasmocytosis to be the only abnormality.

Patient A. P., a 45-year-old Negro woman, possessed a broad heterogeneous gammaglobulin elevation associated with a marked plasmocytosis of the bone marrow. Clinical studies revealed the presence of a small pulmonary lesion which at autopsy 2 months later was shown to be bronchogenic carcinoma with widespread metastases. The patient's marrow aspirate after incubation with tritiated leucine revealed radioactivity corresponding to the gammaglobulin peak. Cell-free plasma incubates did not show radioactivity (Fig. 12).

DISCUSSION

In 1948, Fagreus[12] demonstrated the production of antibodies by the rabbit spleen in vitro. To date this has been the most convincing demonstration of synthesis of gammaglobulin by the reticuloendothelial system since it excludes other possible sites of production. The present method applies a similar principle to the study of human marrow cells in vitro, and additionally utilizes the incorporated radioactive label into protein as a possible indicator of biosynthesis. Grisiola[13] has shown that leucine is a constituent of both normal and myeloma globulin, constituting 10 to 15 per cent of the total amino acid composition. Only serine, aspartic acid, and glutamic acid are present in equal or greater amounts. For this reason, tritiated leucine was selected to insure efficiency of labeling.

There are a number of problems encountered in the use of tritium for tracer studies. These include: (1) the softness of the tritium beta emission, (2) internal molecular radiation of tritiated compounds, and (3) instability of hydrogen atoms in the molecule. Controls were designed to obviate these factors as much as possible. In the experiments described, consistent labeling of myeloma protein, macroglobulin, and of heterogeneous globulin fractions by marrow incubation with H_3 DL-leucine was observed, whereas labeling of proteins in cell-free incubates of plasma treated identically was never observed. Normal marrow cells did not produce measurable radioactivity in the protein in the two controls studied. In two cases of nonmyeloma hypergammaglobulinemia, protein labeling was demonstrated, but to a lesser degree than in multiple myeloma. In material from the patient with macroglobulinemia of Waldenström, radioactivity was noted in both the purified macroglobulin precipitate and the supernatant after penicillamine therapy. Two explanations for this phenomena are suggested: (1) Deutsch,[14] Glenchur and associates,[15] and Isliker[16] have demonstrated

that mercaptans apparently split the macroglobulin molecule with molecular weights of approximately 1,000,000 into smaller units of normal gammaglobulin size with molecular weights of 168,000. It is possible that the SH groups of the penicillamine have resulted in the production of smaller protein molecules corresponding to the size of normal gammaglobulin at the expense of the macroglobulin proteins,[17] so that a reduction of macroglobulin is associated with a corresponding increase in normal gammaglobulin; (2) several types of labeled proteins may have appeared simultaneously during incubation.

Autoradiographs of marrow cells made after varying intervals of incubation revealed radioactivity only in those cells usually associated with protein synthesis: plasma cells, myeloma cells, and lymphoid cells.

The possibility that these data may not reflect biosynthesis of protein by marrow cells must be considered. First the labeling observed may be a nonspecific result of irradiation of the preformed proteins by tritium, or by tritium hydrogen ion exchange. The absence of similar labeling in cell-free incubates effectively eliminates this possibility. Second, the cells in some nonspecific manner might catalyze the transfer of tritium to the preformed protein. This is improbable since normal marrow cells did not produce measureable radioactivity of proteins. Furthermore, autoradiography indicated that radioactivity was limited solely to cells usually associated with protein synthesis (e.g., plasmocytic and lymphocytic cells). Nonspecific diffusion of radioactive amino acid into cells seems unlikely since it would be expected to affect all viable cells similarly. Not only were specific cells required to label protein, but sufficient numbers of cells were needed to produce measureable radioactivity. Technically difficult marrow aspirations which produced blood with few marrow cells did not demonstrate measureable labeling.

Studies utilizing Potter's myeloma mouse tumor indicate a close parallel between in vivo and in vitro labeling of the myeloma protein by myeloma tissue and lend additional support to the validity of the in vitro demonstration of biosynthesis. Further confirmatory studies are in progress. This method appears to offer additional support for the reticuloendothelial origin of globulin and may also serve as a sensitive indicator of a critical cellular function, e.g., protein synthesis and secretion.

The use of the chromatographic scanner for the localization and quantitation of radioactivity in paper electrophoretic strips appears to be an effective method for the demonstration of the phenomena under consideration.

SUMMARY

1. An in vitro method is described which permits the demonstration of protein synthesis by bone marrow cells in diseases characterized by hyperglobulinemia.

2. Biosynthesis of protein was demonstrated by the in vitro incorporation of radioactive DL-leucine into gammaglobulin, macroglobulin, and myeloma proteins.

3. Autoradiography demonstrated cytological labeling of the abnormal cells of multiple myeloma and implicated them directly in myeloma protein biosynthesis.

4. Autoradiography also demonstrated cytologic labeling of lymphoid and/or plasma reticulum cells in Waldenström's macroglobulinemia, which indicates that these cells are involved in macroglobulin synthesis.

5. Whereas normal marrow samples produced no detectable radioactive labeling of protein, autoradiographs revealed cytologic labeling of normal plasma cells.

We are indebted to Dr. William C. Levin for his many suggestions and helpful criticisms and to Miss Lorraine Schlote who prepared the autoradiographs.

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STUDIES ON ERYTHROPOIESIS

XVI. Response to a Single Dose of Erythropoietin in Polycythemic Mouse

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The response to administration of erythropoietin is examined in the spleen of mice previously made polycythemic by blood transfusion. In such animals, erythropoiesis ceases, but is again initiated by injection of erythropoietin. The reticulocyte response is proportional to the dose of erythropoietin between 0.75 and 6 units. The magnitude of the reticulocyte response is not further increased by single doses greater than 6 units, although the duration of the reticulocytosis is lengthened when larger doses are employed. Serial observations on spleens of different polycythemic mice at varying intervals after injection of a single dose of erythropoietin disclose a wave of erythropoiesis sweeping through this organ. A proerythroblast peak at 24 hours is followed by a normoblast peak at 72 hours. A reticulocytosis in the peripheral blood follows and after these reticulocytes mature, no morphologic evidence of the short interval of induced erythropoiesis remains.

IN the last decade, there has been extensive investigation of the nature of the stimuli which induce erythropoiesis and the role played by the hormone, erythropoietin. This work has been summarized in recent review articles by Gordon[1] and Jacobson and associates.[2] Matoth and Ben-Porath[3] have concluded "... erythropoietin is present in erythroid cells. It acts by stimulating the cells to mitotic division." Alpin and Cranmore[4] and Erslev[5] have presented evidence that the site of action of this hormone is confined to the stem cell. In their investigations, animals in a state of active erythropoiesis were employed although Erslev used colchicine to inhibit cell division. We believe that much additional information pertinent to the site of action of erythropoietin and the kinetics of red cell production can be obtained by the use of animals in which erythropoiesis has been suppressed. Such a state has been described by Jacobson and associates[6] in the transfusion induced polycythemic mouse, an animal excellent for this purpose because endogenous erythropoiesis has been suppressed by a mechanism not known to produce any damage to the blood forming tissues. It is the purpose of this study to investigate the sequence of events initiated by the injection of a highly purified extract of anemic plasma, rich in erythropoietin, into such an animal.

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Received for publication Feb. 2, 1960.

MATERIALS AND METHODS

CF No. 1 virgin female mice, weighing 20 to 25 grams, were rendered polycythemic by intraperitoneal injections of washed homologous red cells suspended in saline. These red cells were obtained from the jugular vein of exbreeder females killed by cervical fracture. Heparin was used as an anticoagulant. The red cell suspension was given in 0.5 ml. doses (hematocrit approximately 90 per cent) twice daily for 2 days. Studies were begun 6 days after the last injection of red cells, at a time when the hematocrit of the test animals averaged about 75 per cent and erythropoiesis was suppressed as evidenced by the absence of reticulocytes in the peripheral blood smear. Throughout the period of induction of the polycythemic state and the subsequent investigation the animals were furnished with Rockland mouse pellet diet and water ad libitum.

Hematocrit and reticulocyte counts were performed on blood drawn from one of the tail veins. Blood for hematocrit determinations was collected in heparinized capillary tubes and spun in a microhematocrit centrifuge. Reticulocytes were counted by the direct smear method, using brilliant cresyl blue without counterstain. Determinations were made on the basis of number of reticulocytes per 1,000 red cells counted, except where the values were below 0.1 per cent, 5,000 red cells were counted.

For the purpose of histologic studies, mice were killed by cervical spinal fracture and sections of spleen were prepared. The tissue was fixed in Zenker-formol, embedded in nitrocellulose, cut at 5 μ and stained with hematoxylin-eosin-azure.

A highly purified preparation of erythropoietin* was obtained from plasma of sheep rendered anemic by administration of phenylhydrazine and purified by a series of passages through DEAE† and the Amberlite carboxalate resin XE97. It appears to be a glycoprotein. Its preparation and characterization have recently been described.[7, 8]

The material was dissolved in 0.9 per cent sodium chloride and its potency, as used in these experiments, was determined in units of activity as defined by Goldwasser and White.[7] In the standard starved rat assay for erythropoietin, one unit is that amount which elicits a response, as measured by Fe^{59} incorporation by red cells, equal to that produced by injection of 5 μM of cobaltous ion in the assay animal.

RESULTS

1. *Establishment of Single Maximum Stimulating Dose.*—Polycythemic mice were given single subcutaneous injections (0.5 ml.) to determine the lowest dose, in units of activity, which would produce a maximum response, as determined by reticulocyte count. Serial dilutions from 24 units to 3 units were employed and each dosage group in Table I is represented by an average obtained from 4 animals. Several of the values appearing in Table I are plotted on Fig. 1 which also includes the effect of a single dose of 0.75 units of erythropoietin injected into another group of animals prepared in the same way at a later time.

The peak reticulocyte response was obtained on the third day, regardless of the dose, and the lowest maximum stimulating dose was found to be 6 units of activity. This maximum reticulocyte response persisted into the fourth day in the animals that were receiving the higher doses, but significant variation in reticulocyte response was not observed with dosages of 6 to 24 units in the first 3 days.

*Prepared by Armour and Company Research Division under AEC Subcontract No. 21 under Contract AT-(11-1)-69.

†Diethylaminoethyl cellulose.

TABLE I. PER CENT RETICULOCYTE RESPONSE TO SINGLE INJECTION OF ERYTHROPOIETIN IN POLYCYTHEMIC MICE*

DOSE (UNITS)	HEMATOCRIT ON DAY 0 (%)	DAYS AFTER SINGLE ERYTHROPOIETIN INJECTION					
		0	1	2	3	4	5
24	73	0.0	0.0	0.3	1.4	1.3	0.3
18	71	0.0	0.0	0.4	1.3	1.3	0.3
12	72	0.0	0.0	0.4	1.4	0.9	0.1
9	76	0.0	0.0	0.2	1.1	0.6	0.2
6	70	0.0	0.0	0.3	1.3	0.6	0.2
3	71	0.0	0.0	0.3	0.9	0.4	0.0
Saline control	72	0.0	0.0	0.0	0.0	0.0	0.0

*Each value in this table represents the average obtained from 4 animals.

2. *Morphologic Sequence of Erythropoiesis and Subsequent Reticulocyte Response to a Single Large Injection of Erythropoietin.*—A group of 20 polycythemic mice were given 12 units of erythropoietin on day zero. Four animals were subsequently killed at daily intervals and the spleens were removed for sectioning. Saline injected polycythemic control animals were killed on days 0 and 4 and spleens were sectioned. In all cases, reticulocyte smears of the peripheral blood were made before the animals were killed.

Spleen sections were examined and one spleen section from each day was selected for detailed study. Counts were made of the number of nucleated erythroid cells per 5,000 nucleated cells in the red pulp. Separate counts were made on the red pulp in the center and in a zone within 500 μ of the capsule, since in all sections a greater abundance of nucleated erythroid elements was observed near the capsule.

Erythroid precursors were classified as early or late forms. Counts were made distinguishing between early and later forms of immature nucleated erythroid cells. An early form was considered to be a proerythroblast—i.e., an oval to round cell of about 20 to 30 μ diameter with a large pale nucleus that contained only a sparse amount of fine chromatin. The most important differential characteristic of this cell is a strongly basophilic cytoplasm which stains a strikingly intense blue color. The later form was considered to be a normoblast—i.e., a smaller round cell of about 10 to 20 μ diameter with a more darkly staining chromatin-clumped nucleus, and cytoplasm varying in color from blue to the gray characteristic of a mature erythrocyte.

TABLE II. ERYTHROID CELL COUNTS (PER 5,000 NUCLEATED CELLS) IN POLYCYTHEMIC MOUSE SPLEEN AND PERIPHERAL RETICULOCYTES (PER CENT) AFTER SINGLE INJECTION OF ERYTHROPOIETIN

CELLS	DAY							DAY 4 SALINE CONTROL
	0	1	2	3	4	5	6	
Early Erythroid Cells								
Central	7	37	58	1	11	2	5	6
Subcapsule	12	50	41	1	8	0	6	15
Late Erythroid Cells								
Central	0	0	215	390	24	0	0	0
Subcapsule	0	0	325	570	35	0	0	0
Reticulocytes	0	0	0.3	1.9	1.3	0.4	0	0

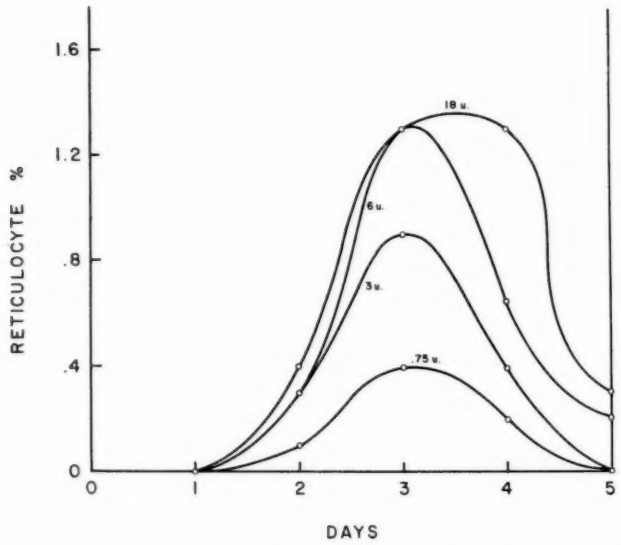

Fig. 1.—Reticulocyte response to a single injection of erythropoietin in the polycythemic mouse.

It was observed (see Table II) that while erythropoiesis was suppressed in the saline-injected control animals, a wave of erythropoietic activity passed through the spleen of the mice injected with erythropoietin. One day after injection, an increase in early erythroid forms was noted, though no normoblasts were observed at this time. There was a striking increase in the number of later forms, i.e., normoblasts on the second day after injection. By the third day, a great abundance of late erythroid cells was present and the reticulocyte count in the peripheral blood had risen to its peak value. By the fourth day, splenic erythropoiesis had fallen precipitously and the peripheral reticulocyte count was only beginning to fall, and on the fifth day, splenic erythropoiesis had returned to its original inactive state and the reticulocyte count was very low but had not yet returned to zero. This sequence, as followed in the subcapsular areas of the spleen and in the peripheral blood is illustrated in Fig. 2.

DISCUSSION

It is our belief that the polycythemic mouse is an ideal subject for the study of many aspects of red cell formation. Here erythropoiesis is depressed by a "physiologic" mechanism, which acts neither as a toxic or nutritional insult to the marrow, nor as an impairment of the metabolic processes of the animal, but rather because the stimulus to erythropoietic activity, erythropoietin, presumably has been removed.[6, 9] If this assumption is correct, then one would expect an orderly and predictable sequence of erythropoiesis to be initiated when this stimulus was again administered. The responses obtained can reasonably be attributed directly to the influences of

erythropoietin: it is not necessary to make any assumptions or corrections accounting for endogenous erythropoiesis. Indeed, the simplicity and reproducibility of the results obtained suggest that these experiments may have widespread applications in areas not intimately related to erythropoiesis. Using the model employed here, many of the fundamental aspects of cell growth, differentiation, and maturation can readily be investigated.

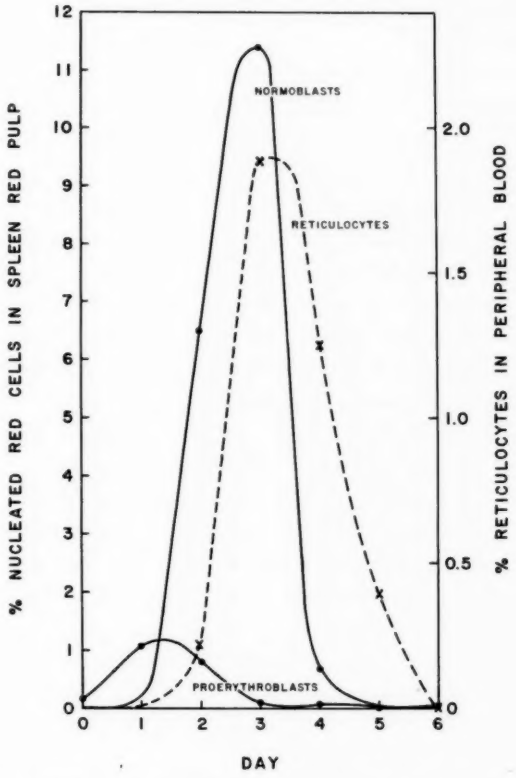

Fig. 2.—Erythropoietic response in spleen and peripheral blood after a single injection of erythropoietin in polycythemic mice.

It is well known that the spleen is an erythropoietic organ in the mouse. The present experiments, insofar as the histologic studies are concerned, have been designed and interpreted on the assumption that changes in erythropoiesis observed in the spleen are representative also of the state of erythropoietic activity in the marrow. Marrow and spleen specimens were examined and compared, though not quantitated, during preliminary studies, and no striking qualitative differences were observed. Spleen is preferred to marrow in these studies only because of the ease with which the spleen can be obtained and the larger amount of readily available hematopoietic tissue it yields.

From Fig. 1 it can be seen that as small a dose as 0.75 unit of erythropoietin elicits a reticulocyte response in polycythemic mice of average weight

23 grams (32 units per kilogram), whereas a single dose of 6 units (260 units per kilogram) produces a maximum response. Although it is realized that marked species differences in responsiveness to erythropoietin may exist, these data are of interest because they indicate the limits within which adequate dosages for clinical testing may lie. It is thus not surprising that we have not yet observed significant response in patients given 600 units daily (10 units per kilogram) of the fractions used in this experiment,[8] since these data suggest this may well be below the minimum dose required to induce a response in a sensitive animal.

In this study, erythropoietic activity has been expressed in the unit as defined by Goldwasser and White.[7] Biologic activity might also be defined on the basis of the smallest amount of material necessary to produce a maximum reticulocyte response in the polycythemic mouse 72 hours after injection. Future investigation will be necessary before the precision and sensitivity of the various bioassay procedures used in defining a unit of erythropoietin stimulating activity can be estimated.

The histologic sequence of response to a single injection of erythropoietin is much as one would expect. An orderly wave of erythropoiesis is seen to pass through the spleen. Using the techniques employed in this study, it is possible to estimate the time intervals involved in the process. In polycythemic mice, even prior to stimulation, one can very occasionally find a cell which so closely resembles a proerythroblast (Table II) that it cannot definitely be identified otherwise, and it has therefore been classified as an early cell in the erythroid series. In the subcapsular area where these cells occur in greater abundance than elsewhere in the spleen, they still account for less than 0.3 per cent of the nucleated cells of the red pulp. After a single injection of erythropoietin, proerythroblasts increase in number within 24 hours, presumably by multiplication of the few proerythroblasts present at the time of injection, by differentiation from primitive stem cells, and by subsequent division. By 72 hours, proerythroblasts are absent. This suggests that the duration of this phase is less than 72 hours. Normoblasts were not found in the 24 hour specimen, but were noted at the 48 hour interval. One would expect that some might be found in specimens examined between the 24 and 48 hour intervals. The peak normoblast concentration is noted at 72 hours and is followed by a precipitous decline, very few being present at 96 hours. This rapid decline is presumed to be the result of their maturation and release from the bone marrow as reticulocytes. The normoblast phase is quickly followed by the reticulocyte phase—a total of less than 4 days' duration. Since it is believed reticulocytes do not divide, this is consistent with an average reticulocyte survival time of 2 days. These data clearly demonstrate that the total time from onset of differentiation to release of the young red cells is, except for a very few cells, less than 96 hours. After maturation of the reticulocytes, no evidences of the wave of erythropoiesis can be distinguished.

There was generally a greater abundance of nucleated erythroid cells near the capsule than in the more central areas. Also at 24 hours, the proerythroblasts

appeared to be arranged in clumps near the capsule, whereas this clumping tendency was not so evident in the central area. We have no information that might suggest any explanation of these conditions.

A question of fundamental interest to us has been the mechanism by which erythropoietin stimulates erythropoiesis. The early work of Jacobson and his associates[6] clearly indicates that erythropoietin is instrumental in inducing differentiation of primitive stem cells. Alpin and Cranmore[4] and Erslev[5] have more recently presented evidence suggesting that this is the only action of the hormone, although Stohlman[10] suggests that more than one mechanism may be involved. It is our opinion that the techniques employed in the present experiments may serve to elucidate this problem, and such investigations are contemplated in the near future.

It is of interest to consider this investigation as it relates to the observations of Linman and co-workers.[11] These investigators believe there are at least 2 erythropoietins, one of which is water soluble, probably protein in nature, and active in augmenting hemoglobin production, while the other is said to be lipid in nature and is thought to be the factor that regulates cell division. They also believe that the latter factor, when acting alone, stimulates the production of cells deficient in hemoglobin. In the experiments reported here, a single preparation, highly purified, glycoprotein in nature, and resembling the first substance described by Linman and coauthors, is capable of activating differentiation and subsequent maturation of red cell precursors from primitive mesenchyme. The standard assay of this material depends on Fe^{59} incorporation of developing erythrocytes of the starved rat.[8] Clearly, this one erythropoietic factor alone is sufficient to bring about both differentiation and maturation and no lipid fraction is required. If another factor is already present in abundance in the animal under study, it does not appear to induce differentiation or stimulate proliferation. Undoubtedly many nutritional factors are required for orderly and optimal erythropoiesis. However, our experiments suggest that one factor, glycoprotein in nature, is sufficient to induce differentiation from the reticulum. Once differentiation is initiated, orderly multiplication and maturation proceeds. The number of new cells produced and the iron utilized, as measured by Fe^{59} incorporation,[8] are proportional to the amount of this factor administered. All of the data we have accumulated to the present time are adequately explained by the existence of the single regulating factor, which we know as erythropoietin.

SUMMARY

The response to a purified preparation of erythropoietin as measured by counts of erythrocyte precursors in the spleen and of peripheral reticulocytes was studied in the polycythemic mouse. After a single injection of erythropoietin, an orderly wave of erythropoiesis was observed to sweep through the spleen. Graded responses were obtained for doses ranging between 0.75 and 6 units. Although the peak reticulocyte count persisted longer after large doses, no higher reticulocyte count was obtained in doses above 6 units. Implications of these observations are discussed.

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PHENYLALANINE TOLERANCE TESTS IN PATIENTS WITH LEUKEMIA

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Abnormal amino acid metabolism occurs in all types of human leukemia as shown previously by quantitative analysis of plasma amino acids. Since the phenylalanine value was found to be elevated in all types of acute and chronic human leukemia, this amino acid was chosen for further investigation. The present study describes the capacity of the patient with leukemia to metabolize a loading dosage of phenylalanine compared to that of normal individuals. The results show that leukemic subjects exhibited a higher plasma phenylalanine level and lower plasma tyrosine level than that of normal subjects after phenylalanine administration and suggest that the phenylalanine hydroxylase system in leukemic subjects may be incapable of meeting the challenge of the phenylalanine load. Higher levels of plasma phenylalanine were reached when L-phenylalanine was fed than when an equivalent quantity was administered as the DL-isomer.

QUANTITATIVE changes of plasma amino acid levels have been reported in patients with leukemia as well as in experimental leukemic rats. Waisman[1] used microbiologic techniques and showed significant increases in phenylalanine, tyrosine, and isoleucine in patients with acute leukemia. Kelley and Waisman[2] presented additional data on the free amino acids in plasma from leukemic patients determined by paper chromatographic microanalysis. These workers found an increase in the glutamic acid, tyrosine, phenylalanine, and "leucine" levels in the blood plasma of patients with acute leukemia. However, patients with chronic lymphatic and the chronic myelogenous leukemias had high levels of glutamic acid, phenylalanine, alanine, and proline. Auerbach and Waisman[3] reported that plasma tryptophan, tyrosine, and phenylalanine levels were found significantly higher in the J strain of rat leukemia. It has also been noted in this laboratory that 302 leukemic rats exhibited high plasma phenylalanine and tyrosine levels. These observations provide additional evidence that abnormal amino acid metabolism also occurs in experimental leukemia. Since plasma phenylalanine was found to be high in all cases of leukemia in human beings, this amino acid was chosen for further investigation. The present study describes the capacity of the leukemic patient to metabolize a phenylalanine load compared to that of normal individuals.

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MATERIALS AND METHODS

The normal subjects for this study were University students who were in good health. Twelve leukemic patients were available for this study. The diagnosis was established by bone marrow evaluation in two acute, four chronic granulocytic, and six chronic lymphatic subjects. The age of the patients ranged between 47 and 81 years of age and all were in good nutritional status. The tolerance tests were done soon after the diagnosis was made before any chemotherapy or other treatment was instituted. All subjects fasted overnight and no food was taken during the test. Blood was drawn for a determination of the fasting phenylalanine levels prior to the administration of phenylalanine. In the first series of experiments, the subject was given orally 0.1 Gm. of L-phenylalanine* per kilogram body weight with 8 ounces of frozen orange juice. In the second series of experiments, DL-phenylalanine* was similarly administered in the amount of 0.2 Gm. per kilogram body weight. Additional blood samples were drawn 1, 2, 3, and 4 hours after the phenylalanine was taken. Blood samples were centrifuged immediately and the plasma was analyzed or stored in a freezer at −10° C. until analysis.

L-phenylalanine in plasma was determined by the bacterial decarboxylation method of Udenfriend and Cooper.[4] It has been shown that the D-isomer does not interfere with the determination. Plasma L-tyrosine was measured by the nitrosonaphthol method of Udenfriend and Cooper.[5]

RESULTS

Table I indicates the average plasma L-phenylalanine and L-tyrosine levels in normal and leukemic subjects prior and subsequent to the ingestion of phenylalanine. Fasting levels of phenylalanine and tyrosine of normal subjects fell well within the ranges reported by other workers[6] using the same methods. The plasma phenylalanine level of the leukemic subjects in the fasting state was significantly higher than that of normal subjects, whereas there was no difference in the plasma tyrosine level between these two groups in the fasting state. Kelley and Waisman[2] reported that plasma tyrosine was high only in patients with acute leukemia but not in those with the chronic disease. In the present investigation, leukemic patients were considered as a single group, and only two patients with acute cases were included. All subjects exhibited a definite peak in plasma level of phenylalanine in one hour after the administration of L-phenylalanine, after which the level declined but remained well above base-line values for 4 hours. The plasma tyrosine level was also increased but to a lesser extent and it remained high for a longer period. In the leukemic patients, the plasma phenylalanine levels were about twice those of the normal subjects at each hourly interval, whereas the plasma tyrosine levels were found to be consistently lower than those of the controls. For ease of comparison these data are presented in graphic form in Fig. 1. The results of DL-phenylalanine administration are also presented in Table I. The peak values for plasma phenylalanine were reached within 2 hours after the administration of DL-phenylalanine, in contrast to the one hour peak when the L-isomer was given (Table I). The rate of disappearance of the amino acid from the blood was also slower with the DL-isomer than with the L form. The extent of the phenylalanine increase in the leukemic patients was considerably less than that

*Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE I. PLASMA PHENYLALANINE AND TYROSINE LEVELS OF NORMAL AND LEUKEMIC ADULTS AFTER PHENYLALANINE ADMINISTRATION

FAST (HR.)	SERIES I (0.1 GM./KG. L-PHENYLALANINE ADMINISTERED)		SERIES II (0.2 GM. DL-PHENYLALANINE ADMINISTERED)	
	NORMAL (6)*	LEUKEMIC (5)*	NORMAL (16)*	LEUKEMIC (7)*
Plasma L-*Phenylalanine (mg./100 ml.).*—				
	1.0 ± 0.2†	1.8 ± 0.3†	1.0 ± 0.1†	1.4 ± 0.1†
1	8.7 ± 1.1	15.9 ± 0.8	4.6 ± 0.4	9.3 ± 1.0
2	7.8 ± 0.8	13.3 ± 0.9	7.3 ± 0.4	10.0 ± 0.7
3	5.2 ± 0.6	11.3 ± 1.4	6.5 ± 0.4	8.4 ± 0.8
4	4.0 ± 0.4	7.7 ± 1.1	5.7 ± 0.3	7.7 ± 1.4
Plasma L-*Tyrosine (mg./100 ml.).*—				
	1.5 ± 0.2	1.5 ± 0.2	1.7 ± 0.2	1.6 ± 0.2
1	2.7 ± 0.2	1.7 ± 0.2	3.1 ± 0.2	2.5 ± 0.3
2	3.5 ± 0.3	2.1 ± 0.3	4.1 ± 0.2	3.3 ± 0.3
3	3.5 ± 0.4	2.4 ± 0.3	4.3 ± 0.3	3.2 ± 0.3
4	3.4 ± 0.4	2.1 ± 0.3	4.2 ± 0.2	3.6 ± 0.5

*Number of subjects.
†Standard error of the mean.

obtained after giving L-phenylalanine. Nevertheless, the plasma phenylalanine levels of leukemic subjects after taking DL-phenylalanine were still significantly higher than those of normal subjects. The plasma tyrosine levels were again found to be lower in the leukemic patients than in the control subjects. Fig. 2 illustrates these data for the DL-isomer.

DISCUSSION

In these studies, the leukemic subjects showed certain definite deviations from normal in their response to load tests of phenylalanine. Although the rate of removal of phenylalanine from blood by leukemic subjects appeared to be the same as normal, initial high plasma levels of phenylalanine were observed. These high values may be due to the fact that the rate of incorporation

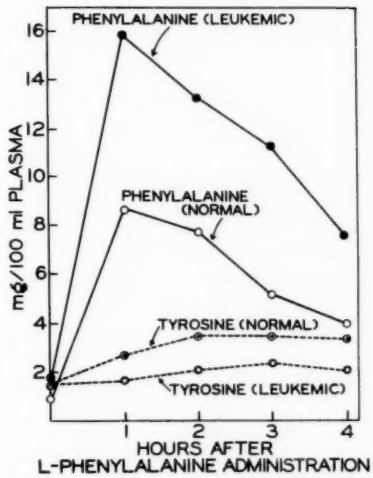

Fig. 1.—Plasma phenylalanine and tyrosine levels of normal and leukemic adults after L-phenylalanine administration.

of phenylalanine into protein of leukemic subjects is slower than normal. This slower rate of phenylalanine incorporation into protein was found to be the case in leukemic rats.* The results also suggest that these subjects have lost some of their ability to metabolize phenylalanine. Since the conversion of phenylalanine to tyrosine by phenylalanine hydroxylase is the main metabolic pathway of phenylalanine it may be that these patients have limited activity of phenylalanine hydroxylase. The consistently lower plasma tyrosine levels of the leukemic subjects after phenylalanine administration would further suggest that the impaired phenylalanine metabolism of these patients is partly due to the loss of activity of phenylalanine hydroxylase. In support of this view, recent preliminary experiments from this laboratory have shown that leukemic rats have a defective phenylalanine hydroxylase system.

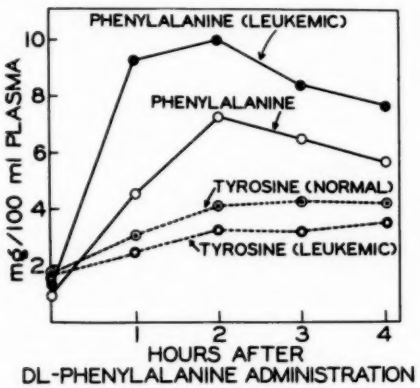

Fig. 2.—Plasma phenylalanine and tyrosine levels of normal and leukemic adults after DL-phenylalanine administration.

In both the L-phenylalanine and DL-phenylalanine tolerance tests, all subjects were given equal amounts of L-isomer per kilogram of body weight. The data indicate that when L-phenylalanine was given, the plasma phenylalanine concentration was higher than when the DL-phenylalanine was given. This effect was more marked in the leukemic than in the normal subject. In addition, there was a tendency for the peak levels to be reached sooner when L-phenylalanine was given. These observations suggest more prompt and possibly more efficient absorption of the L-isomer. Matthews and Smyth[7] have shown that when racemic mixtures of alanine, phenylalanine, or leucine are introduced into the lumen of the intestine of the cat, the L-isomer appears in greater quantities than the D-isomer in the mesenteric blood. However, further study is necessary to establish the inhibiting effect of D-isomer.

SUMMARY

1. The concentrations of phenylalanine and tyrosine in the plasma after the oral administration of DL-phenylalanine or L-phenylalanine have been studied in adult normal and leukemic human subjects.

*Unpublished results.

2. Leukemic patients exhibited a higher plasma phenylalanine level and lower plasma tyrosine level after phenylalanine administration. The results suggest that the phenylalanine hydroxylase system in patients with leukemia may be impaired.

3. The L-isomer of phenylalanine was apparently more efficiently absorbed than DL-isomer.

4. In the leukemic subjects higher levels of plasma L-phenylalanine were reached when L-phenylalanine was fed as such than when an equivalent amount was given in the DL form.

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ORNITHINE CARBAMYL TRANSFERASE ACTIVITY IN HUMAN SERUM IN DISEASES OF THE LIVER AND THE BILIARY SYSTEM

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In man, ornithine carbamyl transferase is localized mainly in the liver and to a lesser extent in the small intestine. Because of these findings, one might expect only damage to the liver or perhaps the small intestine to cause elevated ornithine carbamyl transferase activity in serum (S-OCT).

Serum ornithine carbamyl transferase activity was determined in fifty-six normal subjects and one hundred and eighty-one patients with diseases of the liver or the biliary system. Elevated S-OCT was observed in patients with infectious hepatitis, cirrhosis of the liver, obstructive jaundice, biliary attacks, and cancer of the liver. Patients with silent gall stones, for the most part, had normal S-OCT. Provided widespread disease of the small intestine can be ruled out, elevated S-OCT will specifically point to liver cell damage.

AS REPORTED earlier, ornithine carbamyl transferase[1] in man occurs mainly in liver.[2, 3] Of other organs, only the small intestine contains significant amounts of the enzyme.[3] Increased ornithine carbamyl transferase activity in serum (S-OCT) seems to be almost specific for liver damage and should thus be of diagnostic value.[4, 5]

This study was performed to elucidate the behavior of S-OCT in normal individuals and in patients with disease of the liver and the biliary system.

METHODS AND MATERIALS

Serum Ornithine Carbamyl Transferase Activity.—Serum ornithine carbamyl transferase activity was determined by incubation of serum with citrulline-carbamyl-C^{14} in arsenate buffer, using the method of Reichard and Reichard.[6]* The amount of $C^{14}O_2$ formed is proportional to S-OCT. Results were expressed in micromoles of $C^{14}O_2$ liberated by 0.5 ml. serum per 24 hours incubation under standard conditions.

Other Determinations.—Glutamic oxalacetic transaminase activity in serum and glutamic pyruvic transaminase activity in serum were assayed by the methods of Karmen[7] or Reitman and Frankel.[8] The two methods yielded identical values which are expressed in Karmen units. The icterus index (M) was determined according to Meulengracht.[9] Serum bilirubin was assayed by the method of Jendrassik and Gróf.[10]

Material.—Serum ornithine carbamyl transferase activity was determined in 21 healthy male blood donors and in 35 healthy female members of the hospital staff, all between 20 and 50 years of age. In addition samples were taken from 181 patients with disease of the liver and the biliary system.

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*In an earlier article[6] the concentration of citrulline carbamyl-C^{14} was erroneously given as 1 μM per milliliter. This should be 10 μM per milliliter of final incubation mixture.

RESULTS

Normal Subjects.—The S-OCT of 56 normal subjects ranged between 0.004 and 0.046 μM $C^{14}O_2$ per 0.5 ml. serum, with a mean of 0.018 and a standard deviation of 0.009. The distribution was too skewed to permit calculation of the upper normal limit from the calculated standard deviation. A more normal, though not ideal distribution, was obtained by converting the values into logarithmic form. Since only elevated values would be of diagnostic importance, the border line between normal and elevated S-OCT was calculated according to the formula: border line = antilog $(\bar{x} + s \cdot t_p \cdot /\theta/)$, where $\bar{x}$ equals arithmetic mean; s equals standard deviation (both $\bar{x}$ and s were calculated on basis of log values of original figures). $t_p /\theta/$ was calculated according to Hald.[11]

According to this calculation 95 per cent of all normals will fall with 95 per cent probability below 0.049 μM $C^{14}O_2$ per 0.5 ml. serum. For the sake of simplicity the border line was drawn at 0.050 μM $C^{14}O_2$.

Studies of the Patients.—The results are detailed in Table I. Most of the serial S-OCT determinations were obtained in patients with jaundice. The highest S-OCT value of each patient was used in the tables.

Infectious Hepatitis.—Of thirty-two patients with epidemic or inoculation hepatitis and one with infectious mononucleosis hepatitis, all but one had elevated S-OCT. The highest S-OCT was generally recorded on admission when the patients had been icteric for one to several days (Fig. 1). The values then fell rapidly, often reaching normal prior to normalization of serum bilirubin (Fig. 2). The relation between bilirubin, S-OCT, serum glutamic oxalacetic transaminase, and serum glutamic pyruvic transaminase activity in one illustrative case is shown in Fig. 2. The parallel course of the measured enzyme activities is evident.

TABLE I. SERUM ORNITHINE CARBAMYL TRANSFERASE ACTIVITY IN NORMAL SUBJECTS AND IN PATIENTS WITH VARIOUS DISEASES OF THE LIVER AND/OR THE BILIARY SYSTEM

DIAGNOSIS	NO. OF CASES	S-OCT (μM $C^{14}O_2$/0.5 ML. SERUM)			
		NORMAL ≤ 0.050	SLIGHTLY ELEVATED 0.051-0.080	MODERATELY ELEVATED 0.081-0.160	GREATLY ELEVATED > 0.160
Normal subjects	56	56	–	–	–
Infectious hepatitis	33	1	4	3	25
Liver cirrhosis	42	3	5	14	20
Obstructive jaundice	24				
Malignant disease (11)		1	3	1	6
Choledocholithiasis, chronic pancreatitis, and surgical trauma to the common bile duct (13)		1	2	3	7
Choledocholithiasis without jaundice	3	2	–	–	1
Gallstone	58				
Acute (23)		5	2	6	10
Silent (35)		29	3	2	1
Cancer of the liver (primary or secondary)	17	6	3	3	5
Miscellaneous disease	4	2	1	–	1

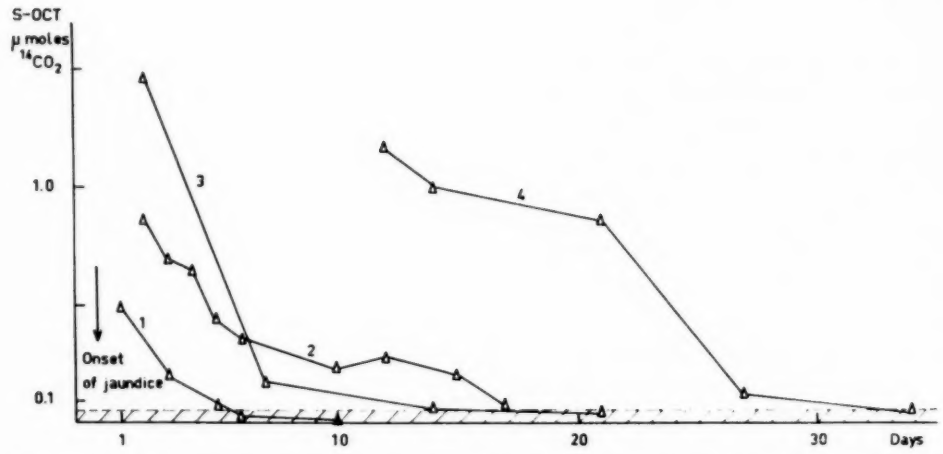

Fig. 1.—Serum ornithine carbamyl transferase activity in four patients with epidemic hepatitis. Shaded area corresponds to normal S-OCT values ($\leq$0.050 μM $C^{14}O_2$).

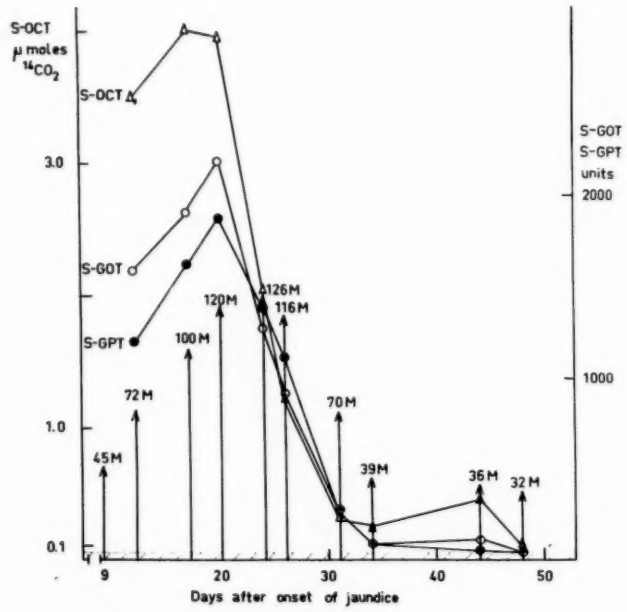

Fig. 2.—Serum ornithine carbamyl transferase, serum glutamic oxalacetic transaminase and serum glutamic pyruvic transaminase in a 74-year-old woman with homologous serum hepatitis in whom samples were obtained before the maximal serum enzyme level had been reached. *M*, Icterus index (Meulengracht). Shaded area corresponds to normal S-OCT.

In one patient (Table II) S-OCT was normal; here the first blood sample was obtained 15 days after onset of the hepatitis. As seen in Fig. 1, S-OCT at this time is often normalized. In some of the patients who did not show complete clinical recovery, the S-OCT remained elevated. One of those cases is illustrated in Fig. 3.

Cirrhosis of the Liver.—Of forty-two patients with clinically proved or microscopically verified cirrhosis of the liver, thirty-nine had elevated S-OCT. As shown in Fig. 4, S-OCT is usually somewhat higher in patients with decompensated cirrhosis (jaundice, ascites, intestinal bleeding, or coma) than in those with compensated cirrhosis. Three cases with normal S-OCT are shown in Table II. Two of these patients were alcoholics with jaundice and ascites and the third was a woman without active disease but with typical biopsy findings.

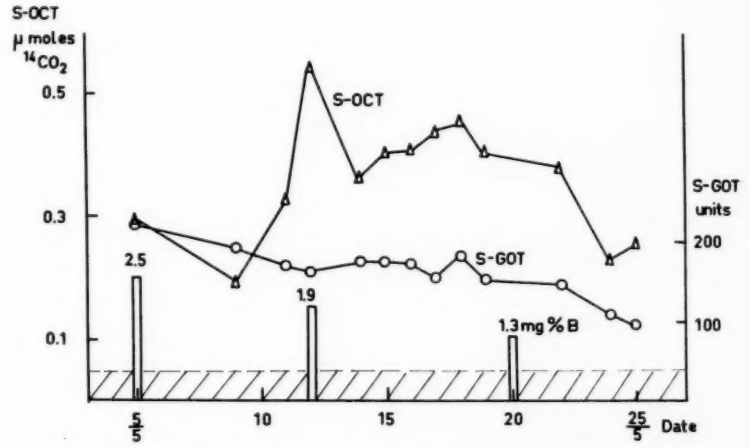

Fig. 3.—Serum ornithine carbamyl transferase and serum glutamic oxalacetic transaminase in a 25-year-old man with epidemic hepatitis who had not recovered 3 months after the onset. *B*, Serum bilirubin in milligrams per cent. Shaded area corresponds to normal S-OCT.

Obstructive Jaundice.—Of twenty-four patients with obstructive jaundice, the impediment to the bile flow was malignant tumor in eleven, choledocholithiasis in eight, chronic pancreatitis in three, and surgical trauma to the common bile duct in two. Elevated values were recorded in all but two patients (Table II). In one of these, in which the jaundice was due to choledocholithiasis and was decreasing, the icterus index was 15. The other patient was a woman with a pancreatic carcinoma. She had been deeply jaundiced for 5 weeks and the serum bilirubin at the time of the test was 17 mg. per cent. Fig. 5 shows the enzyme pattern in a patient with obstructive jaundice caused by cancer of the common bile duct.

Choledocholithiasis Without Jaundice.—Of three patients with choledocholithiasis without jaundice two had normal and one elevated S-OCT (Table II). The latter patient had cholangitis.

Gallstone.—Of fifty-nine patients with gallstones, thirty-five had chronic cholecystitis but no actual symptoms, and twenty-three had acute cholecystitis or biliary colics. All patients were anicteric at the time of blood sampling. Elevated S-OCT was observed in six of the thirty-five asymptomatic patients and in eighteen of twenty-three patients with acute symptoms (Fig. 6).

TABLE II. PATIENTS WITH LIVER DISEASE WITH NORMAL S-OCT, AND ANICTERIC PATIENTS WITH CHOLEDOCHOLITHIASIS AND ELEVATED S-OCT

PATIENT	DIAGNOSIS	AGE (YR.)	SEX	S-OCT (μM $C^{14}O_2$)	REMARKS
I. G.	Infectious hepatitis	25	M	0.044	First blood sample was obtained 10 days after onset of jaundice
G. D.	Liver cirrhosis	60	M	0.015	Chronic alcoholic with jaundice and ascites
A. E.-R.	Liver cirrhosis	65	F	0.035	Chronic alcoholic with jaundice, ascites, and esophageal varices
A. K.	Liver cirrhosis	66	F	0.017	Biopsy verified diagnosis. Palmar erythema, elevated alkaline phosphatase
M. N.	Pancreatic cancer with bile obstruction	64	F	0.038	Deeply jaundiced for 5 weeks. Bilirubin 17 mg. %. No liver metastasis seen at operation. Elevated alkaline phosphatase
A. C.	Choledocholithiasis	73	F	0.049	Receding icterus. Elevated alkaline phosphatase. Icterus index 15
F. P.	Choledocholithiasis with cholangitis	67	M	0.382	Repeated attacks of cholangitis. Greatly elevated alkaline phosphatase but no jaundice
P. P.	Multiple liver abscesses (Salmonella typhimurium)	63	M	0.024	Liver enlargement, attacks of fever. Anicteric, elevated alkaline phosphatase but otherwise normal liver tests. Diagnosed at autopsy

Primary Cancer of the Liver or Metastatic Liver Cancer.—All seventeen patients with cancer of the liver or metastatic liver cancer were anicteric at the time of blood sampling. Six of the patients had normal and eleven had elevated S-OCT. Of the eleven with elevated S-OCT four had normal alkaline phosphatase activity in serum. There were no differences observed in the location, type, or size of cancer between the group with normal S-OCT on one hand and the group with elevated S-OCT on the other. Fig. 7 shows the S-OCT in a patient with pancreatic carcinoma and liver metastasis. S-OCT was elevated both before the onset and during the jaundice.

Miscellaneous Diseases.—Normal S-OCT was found in a patient with malabsorption and in a patient with multiple liver abscesses caused by *Salmonella typhimurium* (Table II). Elevated values were seen in a patient with a liver cyst and a patient with malabsorption and cirrhosis of the liver.

DISCUSSION

An elevated serum level of a primarily intracellular enzyme may be due to an increased inflow of that enzyme into, or a decreased outflow from, blood serum. Our knowledge about the passage of intracellular enzymes to, and their elimination from, the blood stream is very meager.

It has been shown in animal experiments[12-14] that infarcted heart muscle releases glutamic oxalacetic transaminase from the injured cells to the blood. Hence it is assumed that cell lesions must be the usual and perhaps the only cause of intracellular enzyme leakage into blood serum. Conversely, an increased content of intracellular enzymes in blood should imply cell damage if decreased elimination could be ruled out.

Enzymes may be eliminated from blood serum either by inactivation or by excretion in the urine, bile, or other secretions. Ornithine carbamyl transferase occurs in the bile[3] and could be eliminated by this route.

When disease is present, the aim of the clinician is to decide which organ is involved. There is a diagnostic trend to find organ specific enzymes. An elevated amount of such an enzyme in the serum would thus serve to identify the organ affected. Since current liver tests have certain shortcomings, several new methods of enzyme assay such as quantitative measurements of glutamic

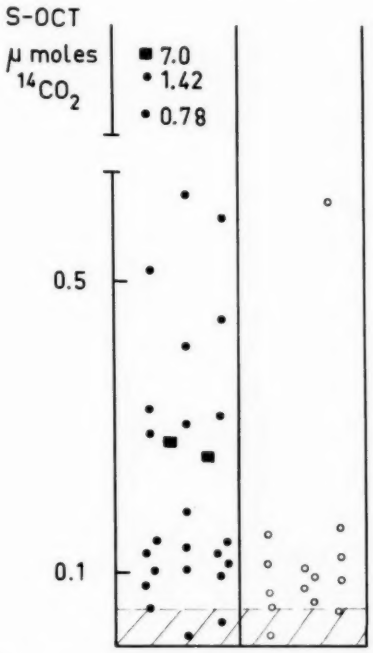

Fig. 4.—Serum ornithine carbamyl transferase in patients with hepatic coma (black squares), otherwise decompensated liver cirrhosis (black circles), and compensated liver cirrhosis (white circles). Shaded area corresponds to normal S-OCT.

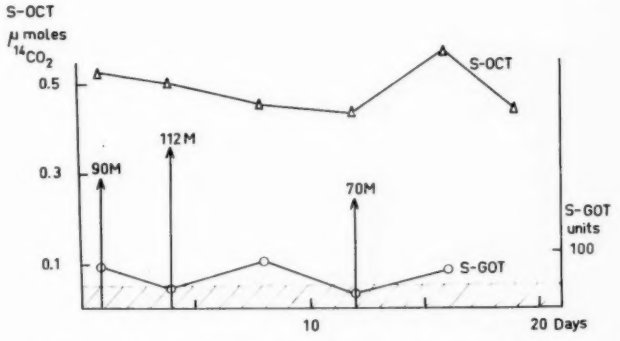

Fig. 5.—Serum ornithine carbamyl transferase and serum glutamic oxalacetic transaminase in a 60-year-old woman with obstructive jaundice for several weeks. *M*, Icterus index. Shaded area corresponds to normal S-OCT.

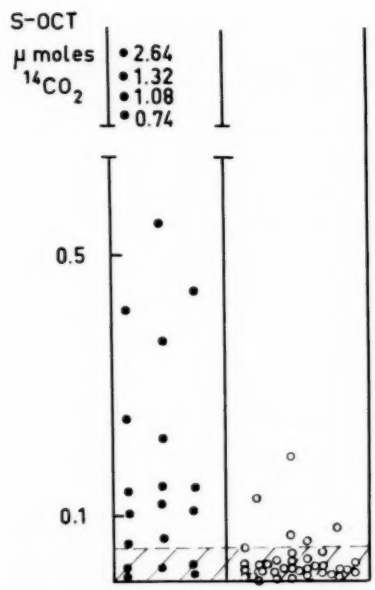

Fig. 6.—Serum ornithine carbamyl transferase in patients with biliary attacks (black circles) and silent gallstone (white circles). Shaded area corresponds to normal S-OCT.

oxalacetic transaminase,[15] glutamic pyruvic transaminase,[16] and isocitric acid dehydrogenase[17] in serum have rapidly gained importance in the diagnosis of hepatic disease. All these enzymes occur abundantly in the liver but are also found in other organs. Therefore extrahepatic cell injuries, too, may produce elevated serum levels.

Citrulline phosphorylase[2] appeared to be an enzyme highly specific for the liver. Krebs and co-workers[2] demonstrated large amounts of it in liver but found none in other mammalian organs tested. After purification and study of the enzyme, the designation, ornithine carbamyl transferase, was suggested by P. Reichard.[1] Preliminary results[4, 5] from patients' sera showed elevated serum ornithine carbamyl transferase activity (S-OCT) only in association with hepatic disease and thus added support to the assumption that ornithine carbamyl transferase was confined to the liver. Subsequent investigation of organ homogenates from dog[18] and man[3] revealed, however, not only large amounts of ornithine carbamyl transferase in the liver but significant quantities in the gastrointestinal tract, notably the small bowel.

On the basis of the ornithine carbamyl transferase activity in human organ homogenates[3] it may be estimated that 1 gram of human liver has approximately the same ornithine carbamyl transferase content as 7 grams of small bowel or 500 grams of stomach or large bowel. Theoretically, therefore, it is only the liver and perhaps the small intestine which by cell damage may give rise to significant S-OCT elevations.

The question as to whether cell death is a prerequisite for the leakage of intracellular protein material and contained enzymes into the blood stream

is not clear cut. Be that as it may, it is of practical clinical significance to be able to decide the connection between certain organic diseases and the serum activity of ornithine carbamyl transferase.

In relation to the normal values, the amount of S-OCT can be divided into normal, slightly, moderately, and greatly elevated values.

All cases of *infectious hepatitis* where blood samples were taken in the beginning of the illness showed increased S-OCT. The amount of S-OCT could be 100 times the normal, and returned to normal values rapidly in uncomplicated cases. On the other hand, in complicated cases the S-OCT remained elevated. Patients with *cirrhosis of the liver* seen in the hospital had, for the most part, an increased S-OCT; a relationship between the patient's liver function and S-OCT was present. Infectious hepatitis as well as cirrhosis of the liver is characterized by liver cell damage. The increased S-OCT which is present in both of these conditions can well be explained by an increased leakage of liver ornithine carbamyl transferase into the blood stream. Probably the role of S-OCT excretion by the gall is not great, for in hepatitis a sharp decrease in S-OCT is seen while the serum bilirubin is still rising.

In *obstructive jaundice*, twenty-two of twenty-four patients had elevated S-OCT. The two cases in which normal S-OCT values were obtained can be explained in that they represent, respectively, a late period of biliary tract obstruction and a receding biliary tract obstruction. From experiments with dogs,[18] it is known that after ligature of the common bile duct, S-OCT rises to maximal values within 4 days, before it decreases again. After restoration of bile passage, a rapid normalization of S-OCT occurs, so that normal S-OCT is present before the serum bilirubin returns to normal values.

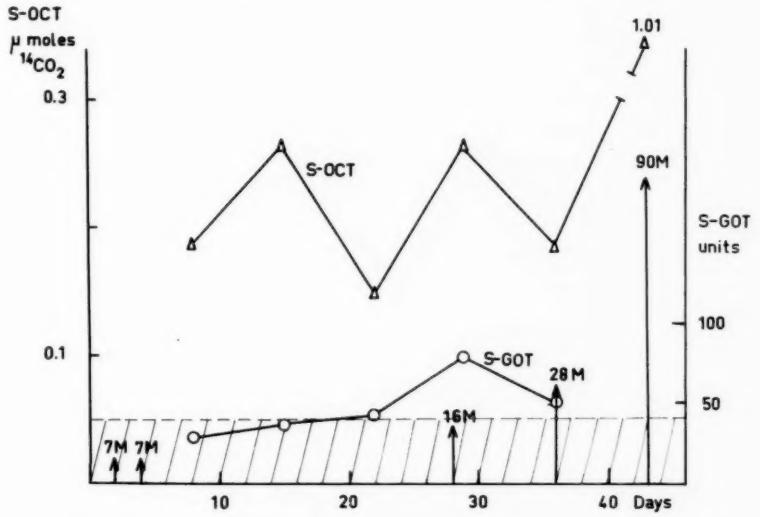

Fig. 7.—Serum ornithine carbamyl transferase and serum glutamic oxalacetic transaminase in a 54-year-old man with pancreatic carcinoma and liver metastasis. Patient was not jaundiced on admission and had rising jaundice from about the twentieth day. *M*, Icterus index. Shaded area corresponds to normal S-OCT.

Acute biliary colic was, like *acute cholecystitis*, often accompanied by an increased S-OCT whereas chronic cholecystitis for the most part had normal S-OCT. Very probably, the elevated S-OCT in this material depended on liver damage caused by passive bile stasis, cholangitis, or possibly a reflux of pancreatic secretions.[19] That six patients with silent gallstones had increased S-OCT might be explained by undiagnosed damage to the liver or a recent and unrecognized gallstone attack.

Of patients with primary or metastatic *carcinoma of the liver*, eleven of seventeen had increased S-OCT. There was no relationship between increased S-OCT values and the type of cancer or the extent of its metastases. It is easy to understand how cancer in the liver could produce bile stasis in some of the small biliary passages and thus produce liver damage with leakage of enzyme into the blood, without causing bilirubinemia. It is, on the contrary, more difficult to explain why some patients with cancer had normal S-OCT.

In a report[20] which will follow, it is shown that most diseases which do not involve the liver do not give increased S-OCT. The exceptions to this are made up primarily of diseases with severe circulatory disturbances which affect the liver, such as congestive heart failure, rapid cardiac arrhythmia, and shock. In addition, certain forms of intoxication and especially those with delirium tremens and acute alcoholic intoxication are accompanied by increased S-OCT. With acute enteritis, about 30 per cent of cases have increased S-OCT, and it is uncertain if this is caused by ornithine carbamyl transferase put into circulation by the cells of the small intestine, or is a result of secondary liver damage.

From the results presented here, it appears that the determination of S-OCT is a sensitive test for damage to the liver, and if damage to the small intestine of greater extent can be ruled out, it is thought to be specific for the liver. The determination of S-OCT ought to be diagnostically valuable in the preicteric phase of hepatitis, in estimating if an acute hepatitis has been cured, in the detection of asymptomatic cirrhosis of the liver, in the diagnosis of anicteric common bile duct stones, and in diseases in which there is a suspicion of involvement of the liver.

Like all laboratory tests, the results of S-OCT elevation should be judged against the background of the other clinical findings. Caution in the interpretation of increased S-OCT is certainly called for, as little is known about the metabolism of ornithine carbamyl transferase in the body.

SUMMARY

As reported earlier, ornithine carbamyl transferase in man occurs mainly in liver and to a lesser extent, in the small intestine.

Ornithine carbamyl transferase activity in serum (S-OCT) was determined in 56 normal individuals and ranged between 0.004 and 0.046 μM $C^{14}O_2$ per 0.5 ml. serum, with a mean of 0.018. Serum ornithine carbamyl transferase was also determined in 181 patients with disease of the liver and/or the biliary tract. Elevated values were observed in nearly all patients with infectious

hepatitis, in most patients with cirrhosis of the liver, obstructive jaundice, and biliary attacks, and in two thirds of the patients with cancer of the liver. Patients with silent gallstones for the most part had normal S-OCT.

If extensive disease of the small intestine can be excluded, determination of S-OCT seems to be a specific and highly sensitive test for liver damage.

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EFFECT ON ISOLATED RAT DIAPHRAGM OF AMMONIUM AND RELATED NITROGEN CONTAINING COMPOUNDS

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By using the glucose uptake of the paired hemidiaphragms of the rat, it was noted that ammonium ion had an insulin-like effect. The insulin-like activity of a number of $-NH_2$ compounds was investigated by studying the glucose uptake and the change in glycogen content evoked by the compounds. Significant augmentation of the glucose uptake was produced at a concentration of 0.001 M by the following compounds: guanidine, L-alanine, L-isoleucine, L-leucine, L-arginine, and L-glycine. At a concentration of 0.01 M ammonium was active.

All of the substances augmenting glucose uptake also evoked a decrease in the muscle glycogen, in contrast to the effect of insulin wihch is known to increase glucose uptake. Several other amino acids not affecting glucose uptake also lowered glycogen.

Guanidine completely reversed the epinephrine (0.1 γ per milliliter) block on glucose uptake. In this respect it resembled the effect of phenethyl diguanide on the diaphragm.

None of the substances tested potentiated the effect of insulin, and guanidine reversed the insulin effect on glycogen.

Normal postprandial sera consistently caused an increase in the glycogen content of the diaphragm. This would refute the hypothesis that insulin-like activity of plasma is due to amino acids.

Stimulation of glucose uptake of the rat diaphragm is thus a property of many substances but insulin was the only substance tested which increased glucose uptake and glycogen content together.

IN the course of assaying the insulin-like activity from a large nonpancreatic tumor[1] using the uptake of glucose by the rat diaphragm, it was noted that insulin-like activity was produced not only by the tumor extracts but also by the extracting media. The activity of the media was traced to the presence of ammonium ion. The following studies are designed as systematic study of the insulin-like activity of ammonium ion and of related compounds containing $R\text{-}NH_2$ grouping.

METHODS

The technique of paired hemidiaphragms of the rat as described by Willebrands and associates[2] was used for the assay. One half of each pair acted as a control, and to the media of the other half was added the test substance. The basic medium was as described

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by Gey and Gey[3] and the incubation was carried out in the shaking machine for 90 minutes at 37° C., pH 7.2, and glucose concentration of 150 mg. per cent. At the conclusion of the incubation the medium glucose was determined by the glucose oxidase method[4] and the tissue glycogen by the method of Good and collaborators[5] using the glucose oxidase method for the measurement. Each experiment included at least five animals and the differences in the glucose concentration of the media between control and test halves were averaged and expressed as ΔG and the differences in the per cent glycogen were expressed as ΔGlyc (per cent wet weight). Serum was obtained from patients and frozen immediately after separation.

RESULTS

Effect on Glucose Uptake.—At a concentration of 0.01 M a number of the substances tested produced a significant uptake of glucose as shown in Table I. At a concentration of 0.001 M significant uptake of glucose was stimulated by guanidine, L-alanine, L-isoleucine, L-leucine, L-arginine, and glycine. None of these effects were as marked as that of phenoformin or insulin previously reported.[6] Histidine was the only substance tested which produced a significant decrease in the glucose uptake.

Effect on Glycogen Content of Diaphragm.—All of the substances which evoked a significant increase in the uptake of glucose of the medium also gave a depression of diaphragm glycogen. In addition, DL-serine and DL-threonine, caused a depression of the muscle glycogen without having any effect on the glucose uptake. L-Valine was the only substance tested which increased the glycogen in a manner similar to insulin but had no effect on glucose uptake.

TABLE I. EFFECT OF ADDING TEST SUBSTANCE TO MEDIUM OF ONE HEMIDIAPHRAGM

	GLUCOSE UPTAKE					GLYCOGEN		
TEST SUBSTANCE	CONCENTRATION MOLAR	ΔG	NO. OF RATS	σD	p	ΔGlyc	σD	p
NH_4Cl	0.01	10.0	5	2.2	< 0.01	−0.018	0.006	< 0.05
Guanidine	0.01	16.0	5	3.5	< 0.02	−0.089	0.026	< 0.05
Guanidine	0.001	7.5	10	2.7	< 0.05	−0.046	0.018	< 0.05
Methylguanidine	0.01	16.0	5	2.7	< 0.01	−0.072	0.013	< 0.01
Methylguanidine	0.001	7.2	5	2.6	> 0.05	−0.026	0.012	> 0.05
Glycine	0.001	2.9	10	1.4	< 0.05	−0.044	0.009	< 0.01
L-Alanine	0.001	6.7	15	1.5	< 0.01	−0.003	0.026	> 0.05
L-Alanine	0.01	4.0	5	1.4	< 0.05	−0.019	0.006	< 0.05
DL-Serine	0.01	3.8	10	2.4	> 0.05	−0.016	0.004	< 0.01
DL-Threonine	0.01	1.7	10	2.4	> 0.05	−0.043	0.010	< 0.01
DL-Threonine	0.001	3.2	5	3.7	> 0.05	−0.041	0.010	< 0.02
L-Valine	0.001	3.6	10	2.3	> 0.05	+0.020	0.005	< 0.01
L-Leucine	0.01	13.0	5	4.0	< 0.05	−0.031	0.009	< 0.05
L-Leucine	0.001	6.2	5	0.9	< 0.01	−0.036	0.011	< 0.05
L-Isoleucine	0.01	14.0	10	1.3	< 0.01	−0.016	0.006	< 0.05
L-Isoleucine	0.001	4.1	15	1.8	< 0.05	−0.038	0.009	< 0.01
L-Arginine	0.01	6.5	5	2.0	< 0.05	−0.035	0.032	> 0.05
L-Arginine	0.001	5.0	5	1.3	< 0.05	−0.034	0.011	< 0.05
L-Histidine	0.01	−13.0	5	2.5	< 0.01	+0.018	0.018	> 0.05
Phenoformin	0.001	14.3	10	1.6	< 0.01	−0.026	0.004	< 0.05
Insulin 5×10^{-3} U./ml.	10	20.7	10	3.9	< 0.01	+0.088	0.012	< 0.01

ΔG, mean difference in media glucose concentration (milligram per cent) between control and test hemidiaphragm; ΔGlyc, mean difference in glycogen (per cent) between control and test hemidiaphragms; $\sigma D = \sqrt{\frac{\Sigma d^2}{N(N-1)}}$

TABLE II. EFFECT OF ADDING SUBSTANCES TO MEDIUM CONTAINING INSULIN

INSULIN CONCENTRATION (U./ML.)	SUBSTANCE			Δ_G	NO.	σ_D	p	Δ_{Glyc}	σ_D	p
5×10^{-3}	Guanidine	0.0001	M	5.2	5	3.1	> 0.05	−0.012	0.006	> 0.05
5×10^{-2}	Guanidine	0.01	M	0.8	5	1.6	> 0.05	−0.168	0.012	< 0.01
5×10^{-3}	Guanidine	0.01	M	22.0	5	1.6	< 0.01	−0.168	0.007	< 0.01
5×10^{-3}	NH_4Cl	0.001	M	0.8	5	1.3	> 0.05	+0.088	0.017	< 0.01
5×10^{-3}	NH_4Cl	0.01	M	− 2.5	5	5.3	> 0.05	+0.002	0.007	> 0.05
5×10^{-2}	NH_4Cl	0.01	M	−11.4	5	2.9	< 0.02	−0.009	0.021	> 0.05
5×10^{-4}	Isoleucine	0.0001	M	1.6	5	0.7	> 0.05	−0.018	0.018	> 0.05
5×10^{-3}	Isoleucine	0.01	M	5.8	5	2.0	< 0.05	−0.071	0.025	< 0.05
5×10^{-3}	L-Alanine	0.01	M	− 5.0	5	1.9	> 0.05	−0.176	0.019	< 0.01
5×10^{-2}	Phenoformin	0.001	M	17.0	5	3.4	< 0.01	+0.029	0.022	> 0.05

TABLE III. EFFECT OF ADDING SUBSTANCE TO MEDIUM CONTAINING EPINEPHRINE (0.1 PER MILLILITER)

SUBSTANCE		Δ_G	NO. OF RATS	σ_D	p	Δ_{Glyc}	σ_D	p
Guanidine	0.01 M	26.0	5	5.6	< 0.01	−0.152	0.016	< 0.01
L-Alanine	0.01 M	3.4	5	1.2	> 0.05	−0.080	0.007	< 0.01
NH_4Cl	0.01 M	10.4	5	3.2	< 0.05	−0.017	0.005	< 0.05
L-Isoleucine	0.01 M	−2.4	5	1.8	> 0.05	−0.037	0.011	< 0.05

TABLE IV. EFFECT OF ADDING SUBSTANCE TO MEDIUM CONTAINING INSULIN (5×10^{-3} U. PER MILLILITER) AND EPINEPHRINE (0.1 PER MILLILITER)

SUBSTANCE		Δ_G	NO. OF RATS	σ_D	p	Δ_{Glyc}	σ_D	p
Guanidine	0.01 M	23.0	5	6.2	< 0.05	−0.139	0.011	< 0.01
L-Alanine	0.01 M	−13.0	5	1.0	< 0.01	−0.029	0.012	> 0.05
NH_4Cl	0.01 M	7.6	5	1.4	< 0.01	+0.026	0.016	> 0.05
L-Isoleucine	0.01 M	−23.0	5	2.7	< 0.01	−0.066	0.009	< 0.01

TABLE V. EFFECT OF NORMAL POSTPRANDIAL HUMAN SERA ON GLYCOGEN CONTENT OF DIAPHRAGM

SERUM DILUTION	Δ_{Glyc}	NO. OF RATS	σ_D	p
10×	+0.021	10	0.006	< 0.01
10×	+0.090	5	0.022	< 0.01
3×	+0.044	5	0.011	< 0.01
2×	+0.021	5	0.004	< 0.01
2×	+0.068	5	0.021	< 0.01
2×	+0.074	5	0.074	< 0.01

Substances which were tested and found to have no effect on either the glucose uptake or the glycogen content were urea, phenylalanine, L-lysine, creatine, creatinine, cysteine, cystine, tryptophane, tyrosine, norleucine, proline, hydroxy-1-proline, ethylamine, methylamine, guanidoacetic acid and guanidopropionic acid, all in concentrations of 0.01 molar.

Interaction With Insulin.—When insulin, at a concentration known to give a near maximum effect on the glucose uptake (5×10^{-2} U. per milliliter), was added to the medium, neither guanidine nor ammonium in contrast to phenoformin caused any further increment in glucose uptake (Table II). Insulin added to the media in physiologic amounts (5×10^{-3} U. per milliliter) did not

potentiate the effect of guanidine, ammonium, L-alanine, or L-isoleucine which were effective in the absence of insulin. The potentiating effect of insulin on the phenoformin response has been previously demonstrated.[6]

Guanidine and L-alanine caused a marked reversal of the insulin effect on glycogen content, whereas ammonium augmented this effect.

Interaction With Epinephrine.—Addition of epinephrine to the medium in a concentration of 0.1 γ per milliliter has been shown to block most of the effect of insulin on the glucose uptake[7] but not to block the effect of phenoformin.[6] As seen in Table III, guanidine actually produced a greater increase in the glucose uptake in the presence of epinephrine, ammonium a similar effect, and alanine none. Guanidine and L-alanine both evoked a greater decrease in the glycogen content of the diaphragm in the presence of epinephrine (Fig. 1).

In Table IV are shown the results of adding the test substance to a medium containing both insulin and epinephrine. Guanidine continues to show the same effects on glucose uptake, whereas the effects of L-alanine and L-isoleucine are sharply reversed.

Effects of Serum.—The effect of postprandial serum on the glycogen difference is shown in Table V where a consistent positive change is noted.

DISCUSSION

Several amino acids and related $-NH_2$ compounds have an insulin-like effect on the uptake of glucose by the rat diaphragm but differ from the effect of insulin in that they produce a decrease in the muscle glycogen. In this respect they are like the hypoglycemic drug phenoformin.[6] The compounds studied which produced an increase in glucose uptake also caused a decrease in glycogen; however, the converse was not true and indeed a much more general property of these compounds is to evoke a decrease in the glycogen. There was no correlation between the magnitudes of these two effects.

Christensen and associates[8] have noted an inverse relationship between the concentration of a given amino acid in the cell and the pK_a of the acid. However, since the effects on glucose metabolism in our experiments showed no relation to pK_a of the substances tested, it is doubtful that the effects on glucose metabolism are related directly to cellular uptake of the substance.

Walaas and co-workers[9, 10] using a Krebs-Ringer buffer reported a significant effect on the glucose uptake of rat diaphragm muscle by hydroxyproline and lysine which was not confirmed in our studies. They ascribed the effect of the amino acid to an increase in permeability of the muscle to glucose.

Walaas and collaborators[9] ascribed the insulin-like activity of plasma to amino acids because the activity appeared in ultrafiltrates. This hypothesis would not appear tenable from the foregoing data since amino acids which increased glucose uptake consistently decreased muscle glycogen, whereas the normal sera produced an increase in muscle glycogen in a manner similar to that of insulin.

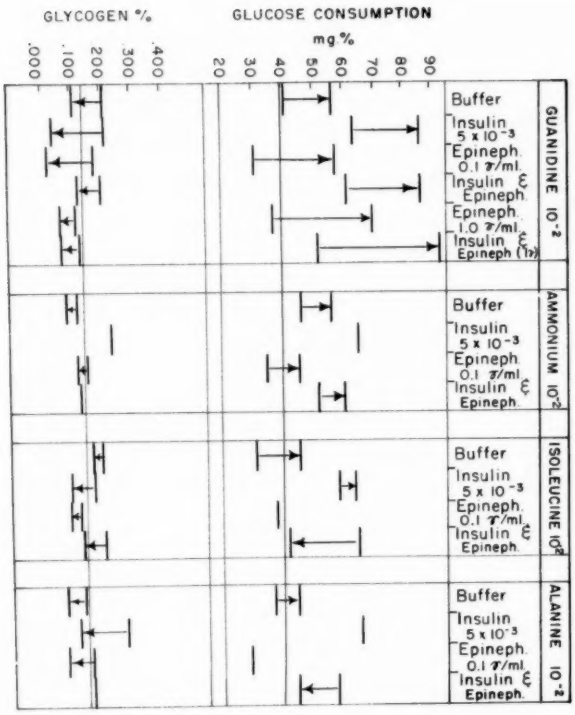

Fig. 1.—The direction and magnitude of the effect of the test substance are indicated by the arrows. The effect on the glucose consumption is shown in the upper part, and the effect on the glycogen content, in the lower.

In the case of phenoformin, this effect may be associated with an increase in anaerobic glycolysis.[11] Dickens[12] demonstrated the ability of the guanidine compounds to inhibit the Pasteur effect.

No effects were noted that would suggest a potentiation of endogenous insulin effect. Guanidine, alanine, and isoleucine decreased glycogen more strongly in the presence of insulin which suggested that they completely canceled the insulin effect on glycogen, in contrast to phenoformin where little effect was produced on the glycogen content under similar conditions.[6]

Guanidine also was able to override the effect of epinephrine on glucose uptake, such that the difference in the glucose uptake between the two halves of the diaphragm was even greater. Thus, part of the effect of guanidine on the glucose uptake may be due to the abolition of endogenous epinephrine effect. The constrictive effects of epinephrine on the other hand have been shown to be potentiated by guanidine[13] which may be related to its effect in inhibiting amine oxidase.[14]

It would not be valid to extrapolate the results of these in vitro studies to the intact organism, but the possibility exists that in certain pathologic states, notably in hepatic or renal failure, the level of amino acids, ammonium, or guanidine may reach values which are high enough to alter glucose metabolism and produce hypoglycemia. The hypoglycemic effect of guanidine has been noted in the rabbit.[15, 16]

It is doubtful that guanidine exists as a free group in any significant concentration normally[17-21] but in renal failure it may reach high levels.[12, 20] An increasing tendency to hypoglycemia is a prominent feature in patients with advanced diabetic nephropathy.[22] The hypoglycemic effects of L-leucine have been described in patients genetically predisposed to this[23] after the ingestion of proteins that contain L-leucine.

SUMMARY

The insulin-like activity of various substances that contain nitrogen was studied by determining the effect on the glucose uptake and the glycogen content of the rat diaphgram. Guanidine, L-alanine, L-isoleucine, L-leucine, L-arginine, and glycine were active in increasing glucose uptake. These substances, as well as DL-serine and DL-threonine, caused a decrease in diaphragm glycogen in contrast to insulin which caused an increase. The effect of the above substances can thus be differentiated from insulin effect by the change in glycogen content.

Application of this technique to human postprandial sera showed that the latter increased glycogen content of the diaphragm which is further evidence for the presence of insulin in plasma.

Guanidine could largely overcome the inhibitory effect of epinephrine on the glucose uptake.

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PLASMA UNESTERIFIED FATTY ACID CONCENTRATIONS IN NEOPLASTIC DISEASE*

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Plasma unesterified fatty acid (UFA) concentrations were measured under standard conditions in normal subjects, in patients with neoplastic disease, and in patients with chronic diseases, other than cancer. Significant increases in plasma unesterified fatty acid concentrations were noted in the group of patients with neoplastic disease. All cancer patients with plasma unesterified fatty acid concentrations above the normal range (0.70 mEq. per liter) had increased neoplastic activity as judged by clinical, radiologic, or laboratory criteria.

In six patients studied more extensively, increases in plasma unesterified fatty acid concentrations appeared to be correlated with disease activity rather than with deficiencies of dietary calories, hypermetabolism, or psychic distress. In one patient with neoplastic disease, exercise coincided with a return of the plasma unesterified fatty acid concentration to the normal range. After this period of exercise, the plasma unesterified fatty acid returned to the previously increased concentration.

DEPLETION of fat stores has been observed in patients and in experimental animals during the growth phase of a wide variety of neoplasms.[1, 2] It may be assumed that during this depletion the lipids are removed from the fat depots and transported to other sites for the respiratory and growth demands of the tumor and host. It has been observed by Gordon and Cherkes[3-5] and Dole[6] that the unesterified fatty acid fraction of the plasma lipid spectrum plays a significant role in lipid transport. Theoretically, therefore, it is possible that changes in plasma unesterified fatty acid concentrations may reflect the rate of depletion of fat depots during the course of neoplastic disease. Plasma unesterified fatty acid concentrations in patients with neoplastic disease have not been reported previously. In this study such plasma unesterified fatty acid concentrations have been compared with those in normal human subjects and in patients with other chronic diseases. The changes in unesterified fatty acid concentrations during the hospital courses of representative patients have been related to other measures of disease activity.

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METHODS

Patient and Subject Material.—All patients with cancer were hospitalized on the Metabolism Service, the Chemotherapy Service, or the Radiation Service of the National Cancer Institute. Malignancy was diagnosed in all patients histologically. There were 19 cases of acute leukemia, 6 cases of chronic leukemia, 9 cases of solid tumor, 4 cases of lymphoma, and 3 cases of myeloma. Normal subjects were members of the professional staff of the National Cancer Institute or hospitalized normal volunteers. Patients with chronic diseases other than cancer were from the National Cancer Institute, from the National Heart Institute, or from Walter Reed Army Hospital. Venipunctures were performed under the same conditions in patients and in normal subjects.

Laboratory.—All blood was drawn between 8:00 and 9:00 A.M. after overnight fasts of 12 to 14 hours except for those rare cases (to be specifically mentioned) in which intravenous solutions were infused during the night. Bloods were drawn from arm veins with tourniquets on the arms into dry syringes and immediately transferred into tubes which contained 0.1 ml. of heparin (100 mg. per milliliter).

The tubes were gently inverted several times and placed in an ice bath. Within an hour, they were centrifuged at 1,500 r.p.m. for 10 minutes. The plasma was then carefully removed from each tube with an individual pipette. A 1.0 ml. aliquot of each plasma was transferred into a 60 ml. glass-stoppered conical bottomed glass tube which contained 30 ml. of an extraction solution composed of 10 ml. of trimethylpentane (isoöctane), 15 ml. glacial acetic acid, and 5 ml. acetic anhydride.[7] A reagent blank was prepared and subsequently treated as a specimen. Plasma proteins were precipitated. Soluble plasma constituents entered the isoöctane or the acetic anhydride–acetic acid phase. Within 24 to 36 hours, as the acetic anhydride combined with plasma water, the 2 phases gradually mixed to produce a homogeneous solution. Within 36 to 72 hours of the start of the extraction procedure, each solution was decanted into an individual separatory funnel, 3 ml. 0.0018N H_2SO_4 was added to each funnel, and the contents were mixed by shaking. The mixture again separated into aqueous-acid and isoöctane phases. The lower aqueous-acid phase was then discarded. The remaining isoöctane phase was washed twice with 25 ml. of 0.0018N H_2SO_4. It was then poured into a 12 ml. test tube, and through it 0.5 Gm. anhydrous Na_2SO_4 was added to absorb any residual wash solution present. Two 4 ml. aliquots from each sample were pipetted into 12 ml. conical centrifuge tubes and titrated with approximately 0.02N NaOH delivered from a calibrated micrometer-type microburette. One milliliter of a freshly prepared solution of Nile blue in ethanol was used as an indicator. The first appearance of a stable pink was used as the end point. During the titration the solution was purged of CO_2 and mixed by N_2 bubbling from a silver needle inserted to the bottom of each tube. The blank titration was subtracted from the sample values and the unesterified fatty acid concentration expressed in milliequivalents per liter. It was assumed that total titratable acidity minus that represented by the reagent blank was for all practical purposes derived entirely from unesterified fatty acids. The standard error of this method is less than 0.01 mEq. per liter between duplicates.

RESULTS

In 30 normal subjects (Fig. 1) the mean plasma unesterified fatty acid concentration was 0.35 mEq. per liter with a standard deviation of 0.12 mEq. per liter. The range was 0.70 to 0.11 mEq. per liter.

Fig. 2 shows a random sampling (first determination) of plasma unesterified fatty acid concentrations of 41 patients with cancer in various stages of the disease superimposed upon the normal distribution. The mean value is 0.58 mEq. per liter with a standard deviation of 0.30 mEq. per liter and a range of 1.53 to 0.15 mEq. per liter. The difference between the means of these two distributions is significantly different ($p = 0.001$). The distribution for the

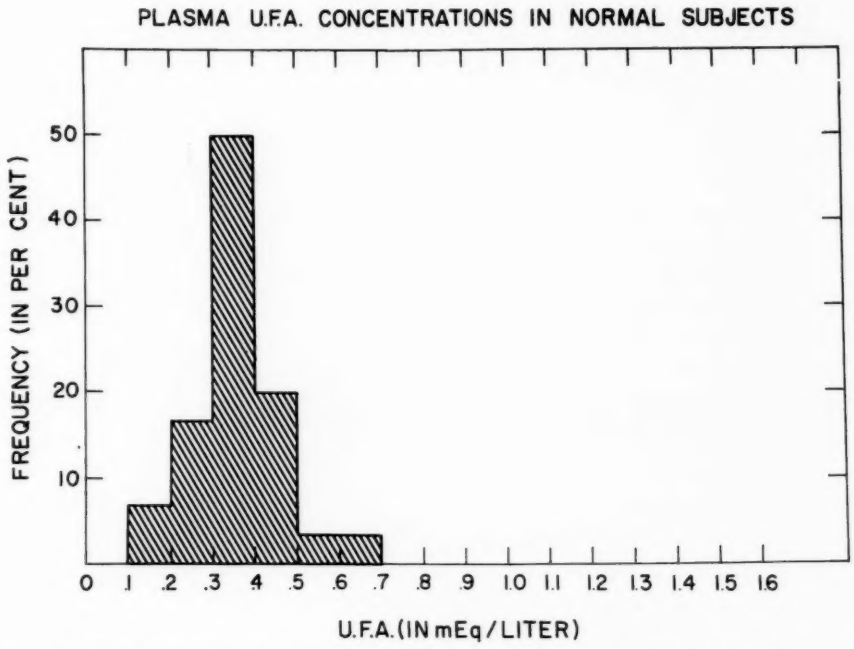

Fig. 1.—Histogram of frequency distribution of plasma unesterified fatty acid concentrations in thirty normal human subjects. Mean, 0.348 mEq. per liter; standard deviation, 0.116 mEq. per liter.

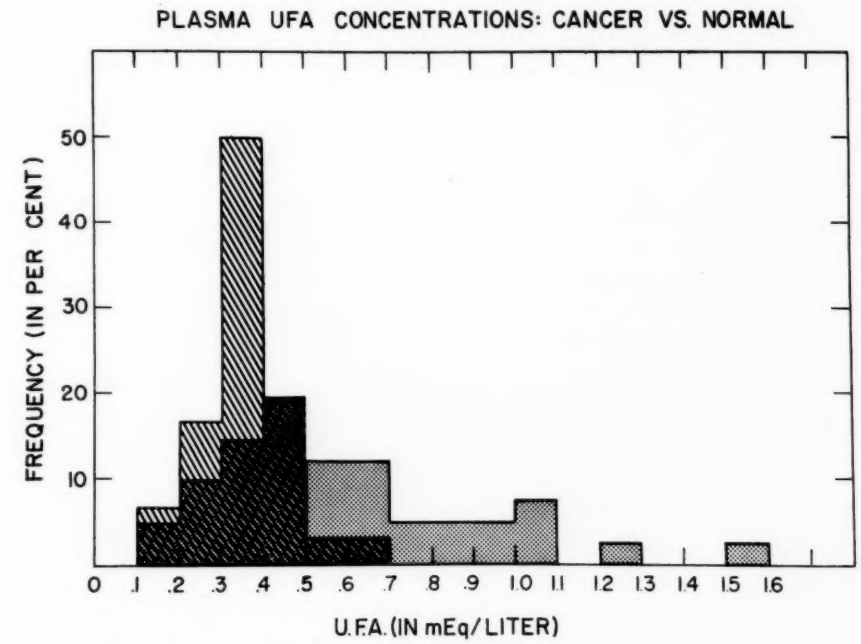

Fig. 2.—Histogram of the frequency distribution of plasma unesterified fatty acid concentrations in forty-one patients with neoplastic disease superimposed on the histogram of the frequency distribution in thirty normal subjects. Mean of patients with neoplastic disease, 0.579 mEq. per liter; standard deviation, 0.300 mEq. per liter.

patients with cancer overlaps the normal subjects' distribution, but 24 per cent or 10 of the 41 patients have concentrations above the highest normal value. The 10 patients with concentrations above 0.70 mEq. per liter had at the time of venipuncture rapidly advancing disease as ascertained by clinical, chemical, or radiologic criteria. Of the 31 patients with concentrations below 0.70 mEq. per liter, 24 patients had inactive disease by the same criteria with or without therapy. Of the 7 with active disease with concentrations below 0.70 mEq. per liter, 3 patients were between 0.60 and 0.70 mEq. per liter, 2 patients died on the day of venipuncture, 1 patient had been recently treated with nitrogen mustard, and 1 patient's plasma unesterified fatty acid concentration rose above 0.70 mEq. per liter within 1 week.

In 18 patients with chronic diseases other than cancer, the mean plasma unesterified fatty acid concentration was 0.36 mEq. per liter. There were 2 cases of Waldenström's macroglobulinemia, 2 cases of idiopathic cryoglobulinemia, 2 cases of pernicious anemia, 4 cases of idiopathic hypoalbuminemia, 3 cases of aplastic anemia, and 1 case each of ulcerative colitis, psychotic depression, postgastrectomy cachexia, rheumatic heart disease with congestive failure, and cachexia due to psychologic causes. It may be seen that despite chronic disease and prolonged bed rest the distribution of the plasma unesterified fatty acid concentrations of these patients is almost identical to the distribution of our normal patients' plasma unesterified fatty acid concentrations and is equally dissimilar to the distribution of plasma unesterified fatty acid concentrations in patients with cancer.

DISCUSSION

From these data two facts appear established. The first is that the patients with cancer *as a group* have statistically higher plasma unesterified fatty acid concentrations than do normal subjects or the patients with the other chronic diseases studied. The second is that the *individual* patients with cancer with elevated plasma unesterified fatty acid concentrations have clinically active disease, and, conversely, those with plasma unesterified fatty acid concentrations within the normal range usually have clinically inactive disease. What remains to be answered is the possible cause or causes of the increase in plasma unesterified fatty acid concentration associated with increased neoplastic activity.

The plasma unesterified fatty acid concentration in a patient may be higher than the concentrations normally noted after an overnight fast for several known reasons. The include poor glucose utilization (as in diabetes mellitus),[8] an infusion of epinephrine,[3] extreme excitement,[9] a fast longer than 18 to 24 hours,* prolonged inadequate dietary intake,[3] hyperthyroidism,[10] a recent injection of heparin,[3] or simple obesity.[6] Growth hormone[11, 12] or ACTH[13] have also been noted to increase plasma unesterified fatty acid concentrations.

None of the patients with neoplastic disease had received heparin, ACTH, or growth hormone, or was obese. None had glucosuria or elevated fasting blood sugar. Protein bound iodines were within normal limits.

*Mueller, P. S.: Unpublished data.

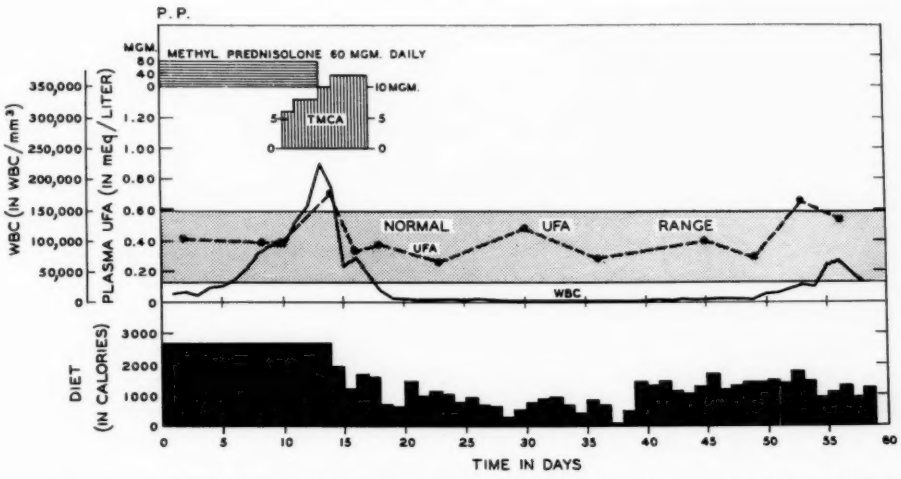

Fig. 3.—Relationships among plasma unesterified fatty acid concentrations, white blood cell counts, and daily caloric intakes during and after therapy with methylprednisolone and trimethyl colchicinic acid–methyl ether–*d*-tartrate (NCI *1136*) in a 38-year-old white man with chronic myelocytic leukemia of two and one-half years' known duration.

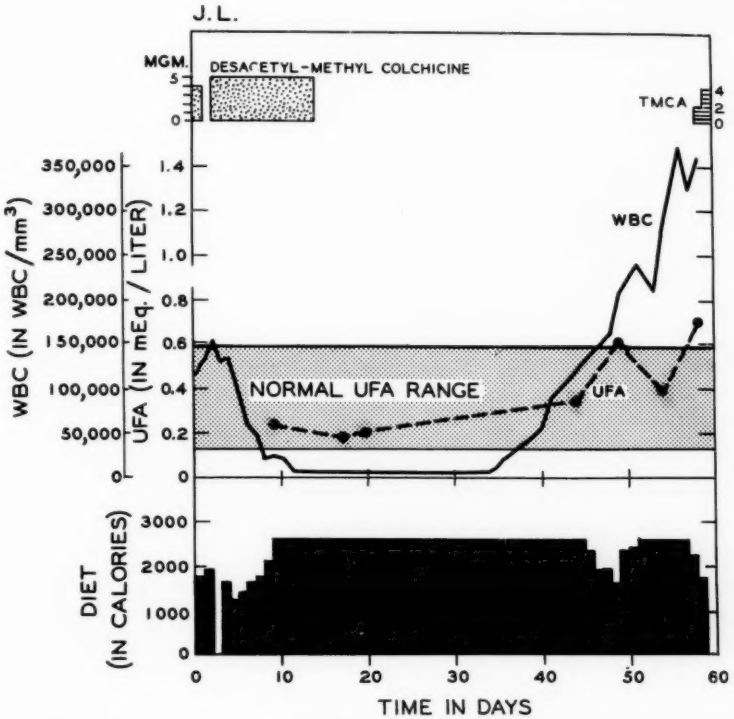

Fig. 4.—Relationships among plasma unesterified fatty acid concentrations, white blood cell counts, and daily caloric intakes during and after or before therapy with desacetylmethyl colchicine and trimethyl colchicinic acid–methyl ether–*d*-tartrate (NCI *1136*) in a 23-year-old white woman with chronic myelocytic leukemia of 15 months' known duration. The omission of a bar representing dietary calories indicates that no accurate estimate of caloric intake was obtained on that particular day.

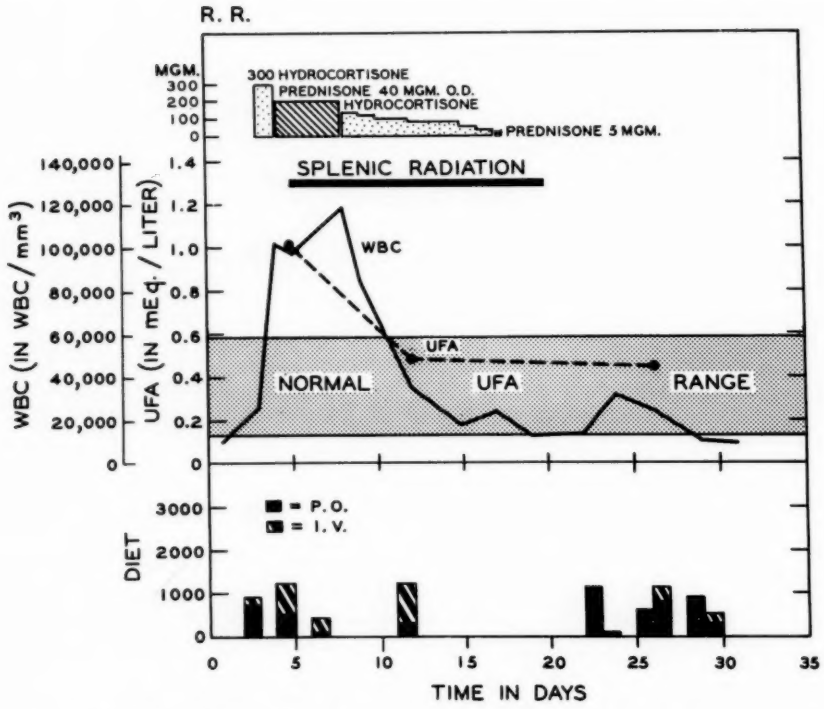

Fig. 5.—Relationships among plasma unesterified fatty acid concentrations, white blood cell counts, and daily caloric intakes during and after therapy with hydrocortisone, prednisone, and splenic radiation in a 30-year-old white man with acute myelocytic leukemia of one year's known duration. The omission of a bar representing dietary calories indicates that no accurate estimate of caloric intake was obtained on that particular day.

The known possibilities that remain are the following: psychic distress, increased metabolic rate, inadequate dietary intake, or any combination of these.

It is conceivable that psychic distress could play some role in increasing plasma unesterified fatty acid concentrations in patients with increased cancer growth. In the studies so far published,[9] plasma unesterified fatty acid elevation has been observed in acute, well-circumscribed, stressful situations. The immediate environmental circumstances at the times of obtaining blood samples were quite similar in all our subjects. It is unlikely that the immediate environmental stimuli selectively operated most effectively only on patients with active tumors. Little is known about the effect of chronic psychic distress on plasma unesterified fatty acid concentration. In a group of 12 patients with chronic schizophrenia, all of whom showed grossly abnormal behavior, and many of whom were chronically anxious, the highest unesterified fatty acid among 22 determinations was 0.44 mEq. per liter.*

Still, it might be argued that the patient with active cancer, sensing the worsening of his condition, might become disturbed enough in some specific way to raise the plasma unesterified fatty acid concentrations. If so, in several instances, they must have sensed rapid tumor growth before the medical staff

*Mueller, P. S.: Unpublished data.

could demonstrate it. It would be necessary to postulate that the patients are able to distinguish rapid tumor growth from other life-threatening illnesses, and that only if they were worsening because of tumor growth would their psychic reactions be of a sort which would cause unesterified fatty acids to increase. The group of chronically ill patients without cancer included a number of patients who were severely ill and who should have had a similar degree of chronic anxiety. Yet not one of these patients had a plasma unesterified fatty acid concentration above 0.55 mEq. per liter. In observing our patients with cancer no increase of morning pulse or blood pressure could be correlated with increased unesterified fatty acid concentrations. Finally, in our survey of patients with cancer, numerous instances were noted of grave clinical deterioration as a result of causes not directly related to increased neoplastic activity such as septicemia, pneumonia, jaundice, fever, or bone marrow failure. In these instances the plasma unesterified fatty acid concentrations were consistently within the normal distribution even though the patient clearly sensed the gravity of his clinical state. Certain of these instances will be cited. The arguments do not completely rule out anxiety as a predisposing factor for increased plasma unesterified fatty acid concentrations in active cancer but certainly do seem to make it highly improbable as the chief cause.

Several of the patients with cancer had increased oxygen consumptions in the basal state. However, no consistent correlation could be made, as a number

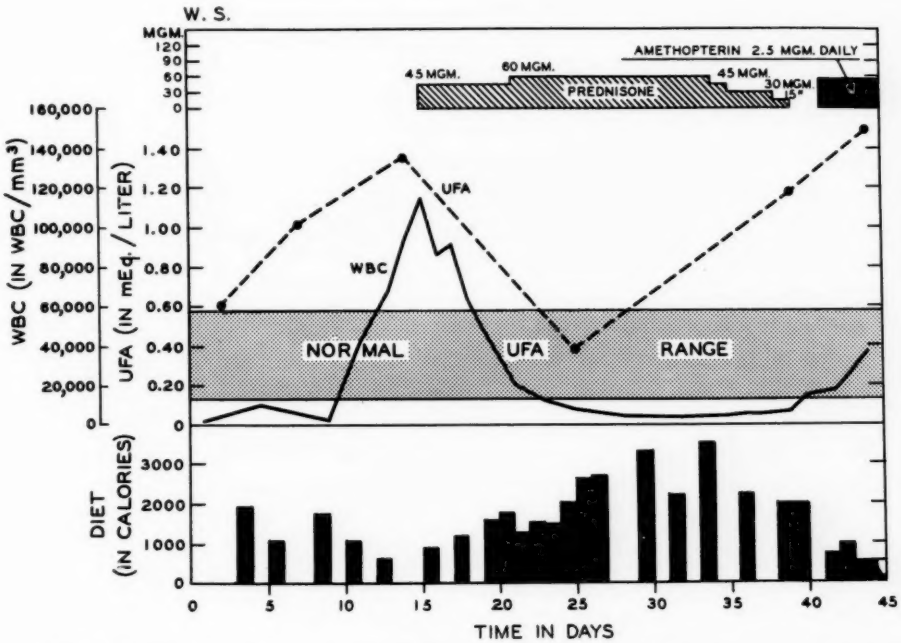

Fig. 6.—Relationships among plasma unesterified fatty acid concentrations, white blood cell counts, and daily caloric intakes before and during therapy with prednisone and amethopterin in a 34-year-old white man with acute lymphocytic leukemia of 6 months' known duration. The omission of a bar representing dietary calories indicates that no accurate estimate of caloric intake was obtained on that particular day.

of increased plasma unesterified fatty acid concentrations were noted despite low oxygen consumptions; and conversely, a number of normal unesterified fatty acid concentrations were noted in hypermetabolic states in such instances as fever and infection.

Frequently the patients in this study had poor caloric intakes on the day before the blood sample was taken. Anorexia is a common symptom and a low caloric dietary intake a common sign in active tumor growth. Theoretically, therefore, the increased plasma unesterified fatty acid concentrations could have been directly related to poor food intake the day before or even the evening prior to venipunctures. Analysis of the data, however, has revealed a number of instances where inadequate dietary intake alone could not account for increased unesterified fatty acid concentrations, whereas increased cancer activity could. Several of these instances will be cited.

Figs. 3 and 4 show the relationships among plasma unesterified fatty acid concentration, dietary intake, white blood cell count, and treatment in two patients with chronic myelocytic leukemia. In Fig. 3 all unesterified fatty acid concentrations were within the normal range (0.1 to 0.59 mEq. per liter) except for 2 separate determinations. The first of these was preceded by an intake of 2,700 calories the day prior to venipuncture with a good caloric intake at the evening meal. The second was preceded by a caloric intake of 1,800 calories with a 1,170 calorie evening meal. Both increased concentrations coincided with the patient's separate white blood cell count elevations. After response to therapy the unesterified fatty acid level returned to the normal range despite a diet lower in calories at the time.

It should be noted that the patient had several febrile periods during which his plasma unesterified fatty acid concentrations were well within the normal range. He was quite ill between the two peaks of increased plasma unesterified fatty acid concentrations with bone marrow failure, secondary to his disease and his treatment with trimethyl colchicinic acid (TMCA), and was well aware of his condition. Therefore, the one single correlation which remains with increased plasma unesterified fatty acid concentration is increased leukemic activity as measured by peripheral white blood cell count. Chronic or acute anxiety, poor food intake, and hypermetabolism do not seem to be significant.

Fig. 4 shows 2 increased unesterified fatty acid concentrations, and the unesterified fatty acid concentrations rose gradually during the course of the patient's illness. The first elevation was preceded by an intake of 1,610 calories and the second elevation was preceded by an intake of 2,230 calories the day prior to venipuncture including an evening meal of approximately 600 calories. These increased unesterified fatty acid concentrations coincided with the elevation of the white blood cell count as in the first case.

Hence, in two patients with chronic myelocytic leukemia, the majority of the unesterified fatty acid concentrations were within the normal range. The increased concentrations noted coincided with increased activity of disease (as judged by white blood cell count) and did not coincide with deficient caloric intake.

Figs. 5 and 6 show two patients with acute leukemia. Fig. 5 shows 1 increased plasma unesterified fatty acid concentration in a patient with acute myelocytic leukemia which coincides with activity of his disease as judged by his white blood cell count. The caloric intake was greater prior to his increased unesterified fatty acid concentration than prior to his 2 normal unesterified fatty acid concentrations. Over the entire period represented in Fig. 5, the patient was gravely ill. Septicemia and jaundice occurred after the tenth day during which time the unesterified fatty acid concentrations were within the normal range.

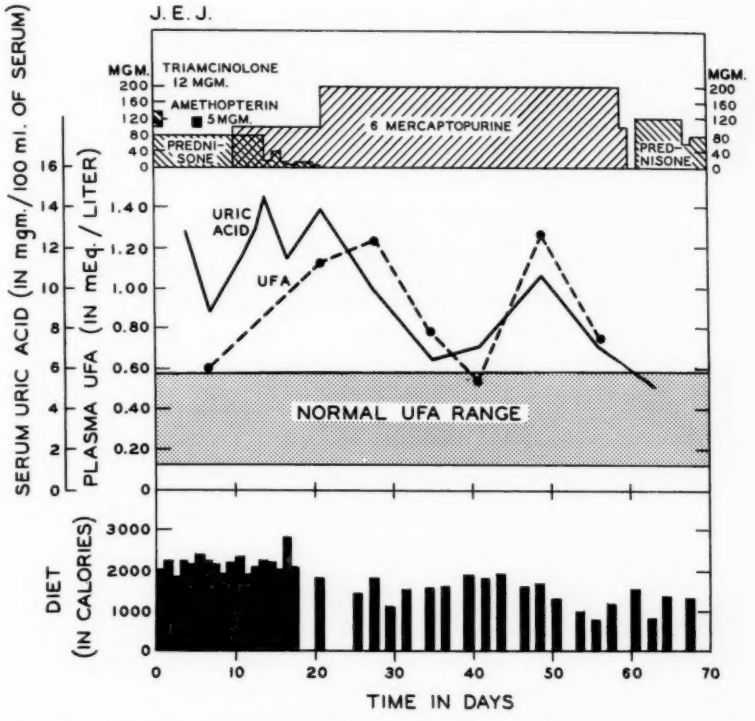

Fig. 7.—Relationships among plasma unesterified fatty acid concentrations, serum uric acid concentrations, and daily caloric intakes during therapy with triamcinolone, prednisone, amethopterin, and 6-mercaptopurine in a 55-year-old Negro man with lymphosarcoma of 1 year's known duration. The omission of a bar representing dietary calories indicates that no accurate estimate of caloric intake was obtained on that particular day.

In Fig. 6, 2 peaks of increased unesterified fatty acid concentrations can be seen during the course of acute lymphocytic leukemia in a patient. These 2 peaks coincide with rising white blood cell count and with poor caloric intake. It should be noted, however, that the caloric intake on the twenty-fourth day was 2,000 calories and the unesterified fatty acid concentration the next morning was 0.37 mEq. per liter. The caloric intake on the thirty-eighth day was 1,995 calories and the unesterified fatty acid concentration the next morning, 1.18 mEq. per liter. These observations reveal that whereas the marked changes in unesterified fatty acid concentrations are related temporally to increased

disease activity and decreased food intake, the increased unesterified fatty acid concentrations precede, rather than follow, decrease in food intake. It should be noted that the first two increases in plasma unesterified fatty acid concentrations occurred prior to white blood cell count elevation and prior to realization by the patient or medical staff of his imminent blastic crisis.

Figs. 7 and 8 depict two patients with solid tumors. In J. E. J. (Fig. 7), a patient with lymphosarcoma with an acute phase of lymphocytic leukemia, the serum uric acid concentration was taken as the best available index of disease activity. The unesterified fatty acid concentrations are positively correlated with the serum uric acid concentrations and are negatively correlated with food intake. Increase in unesterified fatty acid concentration again precedes inadequate caloric intake. The second increase in unesterified fatty acid concentration of the forty-ninth day was preceded by an intake of 1,700 calories. The caloric intake then fell to 800 calories on the fifty-fifth day. The unesterified fatty acid concentration on the morning of the fifty-sixth day was decreased despite the low caloric intake. However, it coincided with the decrease in serum uric acid concentration.

Fig. 8 shows the first admission of a patient with embryonal rhabdomyosarcoma. Increased unesterified fatty acid concentrations were noted despite a weighed, constant daily metabolic diet with a caloric content of more than 60 calories per kilogram per day and a consistently low normal basal oxygen consumption. It should be noted that all increased plasma unesterified fatty acid

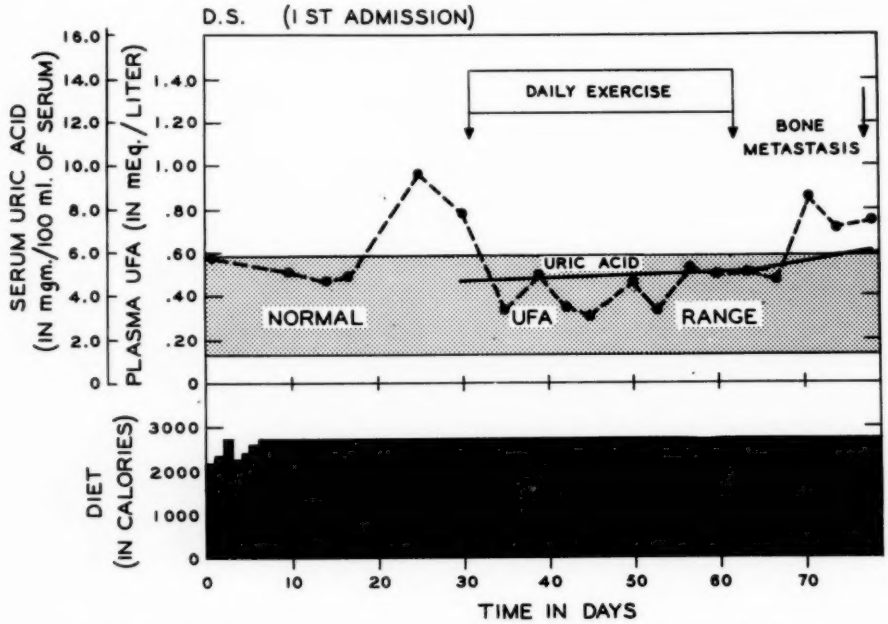

Fig. 8.—Relationships among plasma unesterified fatty acid concentrations, serum uric acid concentrations, and daily caloric intakes in a 15-year-old white boy with embryonal rhabdomyosarcoma of the left testis of 4 months' duration. The patient was exercised one hour daily on a treadmill at 2 miles per hour and a 10 per cent grade from the thirty-second day through the sixty-fourth day.

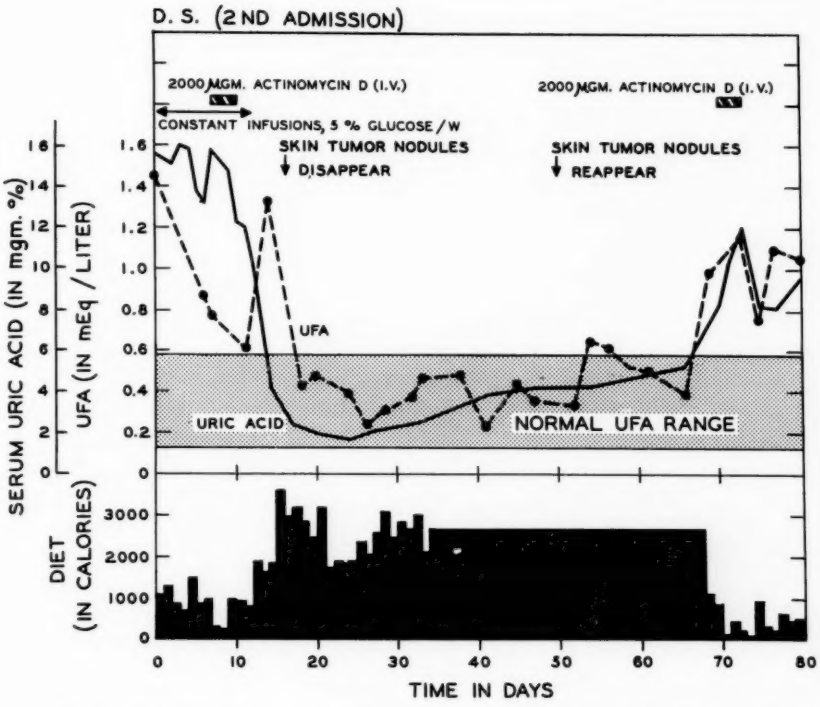

Fig. 9.—Relationships among plasma unesterified fatty acid concentrations, serum uric acid concentrations, and daily caloric intakes in a 15-year-old white boy with embryonal rhabdomyosarcoma of the left testis of 8 months' duration during therapy with Actinomycin D.

concentrations occurred during a period when the patient was entirely well and during which neither the patient nor the medical staff suspected the imminent reactivation of his disease.

During a period of daily exercise on a treadmill, the unesterified fatty acid concentrations returned to within the normal range but again increased on cessation of exercise after one month. The significance of this fall in unesterified fatty acid concentration during a period of time when the patient exercised daily is not now known, but the decrease may be related to enhanced glucose utilization which accompanies exercise as has been noted in the chronically ill by Blatner[14] and in normal subjects by Lutwak and Whedon.[15]

Unlike the other patients, this patient gained weight during the time when his plasma unesterified fatty acid concentrations were increased. This in itself implies an adequate caloric intake. The paradox of weight gain during a period of fat mobilization could be explained by an increased per cent body composition as water as described by Waterhouse and Craig,[16] or as protoplasm, or both.[1, 17]

Finally, Fig. 9 shows the second admission of the patient described in Fig. 8. The markedly increased concentrations of plasma unesterified fatty acid correlated well with serum uric acid concentrations and with decreased food intake. The return of the plasma unesterified fatty acid concentrations to normal was also noted after response to chemotherapy. Attention should be

called to increased concentration on recurrence of disease activity just prior to the onset of anorexia. The brief subsequent fall of unesterified fatty acid after the increased concentration of the fifty-fifth day may be related to the consumption of late evening nourishments delayed because of anorexia and the patient's reluctance to eat at the usual hours. This increase in unesterified fatty acid concentration coinciding with rapid growth of tumor nodules and preceding the onset of anorexia again suggests a relation to disease activity, not to dietary intake.

These studies suggest that plasma unesterified fatty acid concentrations increase during periods of neoplastic disease activity and decrease when the disease responds to various forms of therapy. In several instances these increases in unesterified fatty acid concentrations cannot be related to decreased caloric intake the day or night before venipuncture. In contrast to Mider's observation[18] in the rat with Walker carcinoma 256, in which progressive lipid loss (measured by carcass analysis) did not begin until onset of anorexia, the increase in unesterified fatty acid concentration preceded onset of anorexia by a brief period of time.

The physiologic and biochemical mechanisms responsible for the increases in plasma unesterified fatty acid concentrations in these patients with cancer are not known at present. There may be some relationship to the decreased glucose tolerance and decreased insulin sensitivity noted by Marks and Bishop[19, 20] in the malignant state. However, the patients studied by Marks and Bishop had inactive cancer; we noted normal plasma unesterified fatty acid concentrations in our patients with inactive cancer. A further impairment of glucose utilization might occur with increased neoplastic activity, but Marks and Bishop do not supply the data pertinent to this possibility. Impairment of glucose utilization without obvious laboratory or clinical signs of diabetes might be sufficient to cause increases in plasma unesterified fatty acid concentrations in patients with active cancer.

A second possibility arises in the work of Weil and Stetten,[21] Seifter and Bader,[22] and especially Chalmers and associates.[23] This possibility is that there may be a fat mobilizing substance called forth during rapid tumor synthesis despite a "normal" dietary intake of carbohydrate, fat, and protein and despite a normal basal metabolic rate. Chalmers and co-workers report briefly on two female patients with cancer who had a "fairly normal" food intake. Both of these *nonfasting* patients had higher titers of a fat mobilizing substance (as measured by their mouse liver assay) than the normal *fasting* subjects. More work must be done to define clearly the relationships among anorexia, tumor growth, basal and total energy expenditure, body composition, and plasma unesterified fatty acid concentration.

SUMMARY

1. Plasma unesterified fatty acid concentrations were measured under standard conditions in normal subjects, in patients with neoplastic disease, and

in patients with chronic diseases other than cancer. Significant increases in plasma unesterified fatty acid concentrations were noted in the group of patients with neoplastic disease.

2. All patients with cancer with plasma unesterified fatty acid concentrations above the normal range (0.70 mEq. per liter) had increased neoplastic activity. Conversely, those with plasma unesterified fatty acid concentrations within the normal range usually had inactive disease.

3. In six patients studied more extensively, increases in unesterified fatty acid concentrations were correlated with disease activity rather than with deficiencies of dietary calories, hypermetabolism, or psychic distress.

4. In one patient with neoplastic disease exercise coincided with a return of the plasma unesterified fatty acid concentration to the normal range. After exercise, the plasma unesterified fatty acid returned to its previously increased concentration.

5. Further research is needed to study the relationship of plasma unesterified fatty acid concentrations to other measures of the activity of neoplastic disease.

We are grateful to Miss Helen Noble and Mrs. Ella Greene for their assistance in developing and verifying the accuracy of the unesterified fatty acid method and are indebted to Dr. Edmund A. Gehan, Biometry Branch, National Cancer Institute, for his advice on the statistical analysis of the data.

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MEASUREMENT OF RESIDUAL URINE WITH I^{131}-LABELED DIODRAST

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I^{131}-labeled Diodrast has been used to measure residual urine in the bladder without catheterization in patients with normal and reduced kidney function. Ten to 30 μc of I^{131}-labeled Diodrast mixed with 1 ml. of stable Diodrast has been administered intravenously and after a suitable delay period, radioactivity is counted over the bladder area before and after voiding. The residual urine is calculated from the residual radioactivity in the bladder. In the patients under study, the residual volume measured by the I^{131}-labeled Diodrast method has been checked by catheterization 10 minutes later and a satisfactory correlation found.

MEASUREMENT of the residual volume of urine in the bladder is usually by catheterization which may be traumatic or introduce infection. Either may accentuate the retention and make subsequent measurements difficult to interpret. Even more serious, acute retention may occasionally be precipitated by passing a catheter.

Several methods have been devised to measure the volume of residual urine in the bladder without catheterization. Intravenous radiopaque dye can detect residual urine radiographically,[1] but good kidney function is essential. The phenolsulfonphthalein method,[2] although simple and accurate, also requires normal renal function. The present report describes the use of I^{131}-labeled Diodrast to measure residual urine in the bladder without catheterization. The procedure may be used even in the presence of reduced kidney function.

METHODS

Twenty-eight patients were studied. Eight of the patients had no evidence of lower urinary tract disease and, therefore, were not catheterized. In the twenty patients with evidence or suspicion of lower urinary tract disease, the residual urine was measured (with I^{131}-labeled Diodrast) 5 to 10 minutes before catheterization, except in one patient who was catheterized 2 hours later. Three subjects had indwelling catheters which were clamped to simulate retention. The measurement was repeated in three of the twenty-eight subjects.

I^{131}-labeled Diodrast (Abbott), 10 to 30 μc, mixed with 1 ml. of unlabeled 35 per cent Diodrast solution (Winthrop), was administered intravenously. The patient was allowed food, fluid, and activity ad libitum but was instructed not to void. Radioactivity measurements were made after the subject had acquired the urge to void but not before 1 hour had elapsed in patients with normal blood urea levels and 3 hours in patients with elevated levels.

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A scintillation probe counter with a 2 inch diameter crystal and a broad collimator 2 inches in diameter and 2.5 inches in length was centered over the symphysis pubis of the patient in the supine position with the end of the collimator 20 cm. from the skin. Successive 2 minute counts of the radioactivity over the bladder area were taken. The patient then assumed the erect position, voided into a graduated glass cylinder, and immediately resumed the supine position for measurement of the residual radioactivity over the bladder area. The difference between the pre- and posturination counting rates over the bladder is the radioactivity that was voided. This radioactivity divided by the volume of voided urine gives the counts in each milliliter of urine. From the residual radioactivity in the bladder, the residual volume in milliliters can be calculated. In twenty of the patients, the residual volume was measured by catheterization within 5 to 10 minutes after the I^{131}-labeled Diodrast measurement.

Because Diodrast is not completely excreted during a test, correction was made for the radioactivity measured over the bladder area but not in the urine. The counting rate over the precordium was found to approximate the "background" counting rate and was subtracted from the pre- and postvoiding counts taken over the bladder.

After the initial experiments, 10 to 15 μc I^{131}-labeled Diodrast was found to be adequate. The precise amount of administered radioactivity need not be known. The waiting period of 1 to 3 hours was used to ensure that only negligible amounts of radioactivity were excreted into the bladder between the pre- and postvoiding bladder counts.

RESULTS

In Table I are recorded 10 measurements in the eight patients without evidence of lower urinary tract disease and who were not catheterized. Of these, three had renal disease with elevated blood urea nitrogen levels. The calculated residual urine volumes were all less than 25 ml. and within the normal range.[3] In two instances where the calculated residual volume exceeded 20 ml., extenuating circumstances were present. One subject had not yet acquired the urge to void at the time the test was performed, and the second had a markedly distended bladder and voided 990 ml. Either condition may give rise to increased residual urine volume.[3]

TABLE I. MEASURED RESIDUAL URINE VOLUME OF PATIENTS WITHOUT LOWER URINARY TRACT DISEASE

DIAGNOSIS	AGE (YR.)	BLOOD UREA NITROGEN (MG. %)	URINE VOIDED (ML.)	RESIDUAL (DIODRAST-I^{131}) (ML.)
Rheumatic heart disease, inactive	35	10	373	11
			110*	23
			243	4
Cerebral vascular accident, female	76	29	138	5
Anxiety neurosis	27	15	990	24
Peptic ulcer	35	18	85	1
Chronic glomerulonephritis	32	25	275	6
Rectal abscess, postoperative	43	12	512	17
Chronic glomerulonephritis	47	57	310	11
Peptic ulcer	30	13	490	10

*No urge to void.

In Table II are listed the measurements of residual volume both by this method and by catheterization. Of these patients nine had elevated levels of

TABLE II. MEASURED RESIDUAL URINE VOLUME OF PATIENTS WITH LOWER URINARY TRACT DISEASE

DIAGNOSIS	AGE (YR.)	BLOOD UREA NITROGEN (MG. %)	URINE VOIDED (ML.)	RESIDUAL URINE	
				DIODRAST-I^{131} (ML.)	CATHETER (ML.)
Benign prostate hypertrophy	62	18	295	248	245
Benign prostate hypertrophy	75	17	94	18	13
			65	7	7
Bladder neck contracture	21	17	275	44	51
Fractured femur, benign prostate hypertrophy	69	12	57	51	49
Benign prostate hypertrophy, indwelling catheter	78	18	58	4	0
Benign prostate hypertrophy, indwelling catheter	71	50	95	14	7
Benign prostate hypertrophy	77	25	80	13	22
Benign prostate hypertrophy	62	16	280	17	18
Benign prostate hypertrophy	85	31	135	37	46
Benign prostate hypertrophy	64	19	560	141	140
Diabetes mellitus with Kimmelstiel-Wilson syndrome benign prostate hypertrophy	65	50	380	24	29
Nephrosclerosis, ? benign prostate hypertrophy	66	33	154	7	7
Benign prostate hypertrophy	67	12	425	560	440
Bladder neck contracture	33	21	177	133	190
Benign prostate hypertrophy	64	—	53	2	5
Hypertension	66	25	180	59	77
Benign prostate hypertrophy, diabetes mellitus	67	22	56	59	61
Benign prostate hypertrophy, hypertensive cardiovascular disease	65	18	335	5	40
Arteriosclerotic heart disease, benign prostate hypertrophy	68	68	38	427	410
Benign prostate hypertrophy, diabetes mellitus	59	—	—	1,178	1,300*

*Catheterization was done 2 hours after I^{131}-labeled Diodrast measurement.

blood urea nitrogen. Correlation between the two methods for clinical purposes was good in all but one patient. In this patient, the concentration of counts in both the voided and the catheterized specimen were measured in a well-type scintillation counter and found to differ markedly; the catheterized specimen had only $\frac{1}{4}$ the radioactivity per volume of the voided specimen. This would indicate failure of mixing of bladder urine or, more likely, a diuresis during

the interval between the radioactivity measurement and the catheterization. In one patient, in whom the procedure was repeated after one week, agreement was good.

No untoward reactions to the Diodrast were noted.

DISCUSSION

Winter[4, 5] and others[6] have used I^{131}-labeled Diodrast to study the blood flow and tubular function of both kidneys simultaneously. No reports on its use to measure residual urine have appeared.

The present method seems to be useful for measuring residual urine without catheterization even when kidney function is abnormal. Because Diodrast is rapidly excreted, repeated measurements are not difficult, an important consideration in several types of patients. Routine isotope measuring equipment may be used. It is possible that the material could be given by intramuscular injection. The small amount of Diodrast needed and the rapid excretion of radioactivity make sensitivity reactions and radiation hazards negligible. It can be computed that even if 15 μc of radioactive Diodrast were excreted into the smallest urine volume of this series, the urinary tract mucosa would get less than 0.06 roentgen equivalent physical per hour during the time that the material was retained. Unless it were complete, urinary retention would not prevent the elimination of the radioactive material within a period of less than a day. The radiation would be much less than that given the thyroid in a radioiodine uptake study with an equal quantity of radioactivity.

There are a few limitations to the method. It cannot, of course, be applied in patients with known Diodrast sensitivity, but other compounds such as Urokon, Miokon, and Hypaque are available in radioiodinated form, and might be substituted. There might be some difficulty in getting reliable results in patients wth severe urinary frequency because they would void before the necessary hour had transpired.

The method has several potential sources of error, none of which seem serious. Of these, one which would give falsely high values would be the excretion of radioactivity into the bladder during the time between voiding and the final measuring of bladder radioactivity. Studies of the excretion of I^{131}-labeled Diodrast after intravenous injection show that after 1 hour in patients with normal levels of blood urea and 3 hours in those with reduced kidney function, the radioactivity excreted in this short interval is usually insignificant. Nevertheless, this is always checked by successive measurements over the bladder before the patient voids.

The incomplete excretion of radioactivity during the test necessitates the correction for radioactivity measured over the bladder area but not in the urine. The use of the counting rate over the precordium as background is not highly accurate. In the catheterized patients it was usually lower than the count over the empty bladder but, as evident from the results, it has proved to be adequate.

SUMMARY

The use of I^{131}-labeled Diodrast as a measurement of residual urine in the bladder without catheterization has been found to be of value in patients with *normal* or *reduced* kidney function. The method is safe, reliable, and simple.

We wish to thank Dr. Joseph A. Camilleri, Resident in Urology, for his encouragement, cooperation, and advice.

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PULMONARY VASCULAR CHANGES AFTER INTRAVENOUS INJECTION OF THROMBOPLASTIN IN RABBITS

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This study was designed to determine the pulmonary vascular changes in rabbits after intravascular clotting in the peripheral venous circulation. Two series of 20 rabbits each were given intravenous injections of equal amounts of homologous thromboplastin at regular intervals for a 6 month period. One series received thromboplastin which had a high clotting activity and the other series of rabbits was given material which had been deactivated by the method of Quick. Some animals died shortly after injection of the material after an anaphylactic type of reaction, the remainder were killed after 27 injections.

Changes in the pulmonary vascular bed were then evaluated by the double blind technique and graded by the pathologist before the key was broken. Rabbits receiving active material showed significant intraluminal changes consisting of thrombus formation, re-endothelialization of the thrombus, and proliferative intimal fibroplastic reaction which is later seen as plaques of intimal fibrosis. The findings of this study confirm the reports of others using similar techniques and reaffirm the possibility that intravascular clotting may be one step in the development of certain types of arteriosclerosis.

INCREASED interest brought about by the availability of more precise methods for the study of the pulmonary vascular bed has resulted in a marked increase in the literature concerning pulmonary hypertension.[4, 7, 9, 10, 16, 19, 33, 36, 40, 47] The occurrence of pulmonary hypertension in association with congenital or acquired defects of the cardiopulmonary system has been well recognized for some time. It has become well accepted that in this group pulmonary arteriosclerosis is a secondary change. The changes seen in the absence of primary lung or heart defects have been looked upon somewhat skeptically as being "primary" in the pulmonary vessels. Whether or not these two groups of patients have a common cause for their arteriosclerotic changes has not been established.

Having studied two patients with marked pulmonary hypertension who had histories of obstetrical difficulties, we considered the possibility of a relationship between the two.[17] In 1941 Steiner and Lushbaugh[39] described 8 causes of sudden death associated with amniotic fluid emboli. Studies carried out on rabbits and dogs using human meconium[39] resulted in sudden death of

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most of the animals. The possibility of amniotic fluid entering the circulation suggested that this might provide emboli other than chorionic villi as well. It is known that postpartum hemorrhage has been associated with depletions of fibrinogen[6, 12, 26, 32] in the maternal circulation and this in turn has been thought to be related to the very high thromboplastin activity of amniotic fluid.[34, 44] If small leaks of amniotic fluid occur incident to abnormal implantation of the placenta or manipulation during delivery, intravascular clots might form which would then be filtered out by the pulmonary bed. Extensive studies have been done demonstrating that substances such as fibrin, paper fibers, lycopodium spores, etc., are filtered out by the pulmonary bed. These studies, however, did not involve intravascular clotting. In order to establish the possibility that intravascular clots would be filtered off before their removal by fibrinolysis,[38, 34] small amounts of homologous thromboplastin were injected into rabbits using both active and heat inactivated material. In similar studies Barnard found that changes of pulmonary arteriosclerosis were produced by injections of homologous thromboplastin.[1, 2] The present report confirms these findings.

MATERIALS AND METHODS

A total of 40 female rabbits weighing between 4 and 5 kilograms, all obtained from the same source, were used in this study. Twenty rabbits were placed in the control series and 20 were given injections of active rabbit thromboplastin. The material for injection was made by grinding fresh rabbit brain and extracting it with acetone. The residue was dried, powdered, and stored in a dehydrated container in a freezer. A fresh suspension was made by the following method: 250 mg. of dry material was added to 5.0 ml. 0.15N saline solution containing 0.1 ml. 0.1M sodium oxalate. Gross particles were then removed by filtering the suspension through washed sterile gauze, and tested for thromboplastic activity. The clotting time of an equal mixture of the brain suspension, normal human citrated plasma, and 0.025M calcium chloride was always less than 16 seconds. The material used on the control animals was prepared in the same manner but was inactivated by heating to 60° for 15 minutes according to the method of Quick[30] and then tested for thromboplastic activity and found to produce no clot.

TABLE I. DOSAGE SCHEDULE OF ACTIVE AND INACTIVE THROMBOPLASTIN

RABBIT NO.	INJECTION NO.		
	1 (ML.)	2 (ML.)	3 TO 27 (ML.)
Active material			
1, 2, 3, 4, 5	0.5	0.5	Died after second injection; anaphylactoid death
6, 7	0.5	0.5	0.1 For a total of 27 injections
8, 9, 10, 11	0.4	0.2	0.1 For a total of 27 injections
12, 13, 14, 15, 16	0.3	0.2	0.1 For a total of 27 injections
17, 18, 19, 20	0.2	0.1	0.1 For a total of 27 injections
Inactive material			
A, B	0.5	0.4	0.1 For a total of 27 injections
C, D, E	0.4	0.4	0.1 For a total of 27 injections
F, G, H	0.4	0.2	0.1 For a total of 27 injections
I, J	0.4	0.2	Died of mucous enteritis
K, L, M, N, O, P, Q, R, S, T	0.1	0.1	0.1 For a total of 27 injections

All animals except those noted as dying after the injection were killed after the twenty-seventh injection.

TABLE II. PATHOLOGIC FINDINGS AS NOTED BEFORE SPECIMENS WERE IDENTIFIED AS HAVING RECEIVED ACTIVE OR INACTIVE THROMBOPLASTIN

ANIMAL NO.	DEGREE OF INTRALUMINAL CHANGE	CHANGES IN VESSEL WALL	SIZE OF VESSEL	NO. OF INJECTIONS
Experimental animals				
1	++++	+++	SML	2
2	+++	+++	S	2
3	++++	++++	S	2
4	++++	++++	LS	2
5	+++	+++	S	1
6	+++	+++	L	15
7	++	++	S	13
8	+++	++	S	27
9	++++	0	LS	27
10	++++	0	S	2
11	++	0	S	1
12	++	0	S	27
13	+++	0	L	27
14	+	0	S	27
15	++	0	LS	8
16	++++	0	S	1
17	++++	+	LMS	8
18	0	0	—	27
19	++	0	L	27
20	+++	0	LS	12
Control animals				
A	0	0	—	27
B	0	0	—	27
C	0	0	—	27
D	0	0	—	27
E	0	0	—	27
F	0	0	—	27
G	0	0	—	27
H	0	0	—	27
I	0	0	—	1
J	0	0	—	2
K	0	0	—	27
L	+	0	—	27
M	+	0	—	27
N	0	0	—	27
O	+	0	L	7
P	+	0	LS	27
Q	+	0	L	27
R	+	0	L	27
S	0	0	—	27
T	0	0	—	27

The material was injected usually once weekly into ear veins with a tuberculin syringe and a 26 gauge needle, using about a minute to inject the larger doses and 20 to 30 seconds to inject the smaller doses. It was found that more rapid injections of the larger doses were followed by convulsive seizures and death in what appeared to be an anaphylactoid reaction. The schedule of injection is noted in Table I. All animals were killed within a week after the last injection.

The animals were all given a code number at the beginning of the study and at the time of sacrifice the organs were examined without reference to the type or number of injections that had been given. Representative sections were obtained from each lobe of lung in all animals and were stained with Hematoxylin and eosin. A blind study was then made of the pathologic changes and the degree of change graded before the key was broken. The results of this study are shown in Table II.

RESULTS

By the methodology used in these experiments it was evident that thrombus formations can be produced very rapidly, as they were observed in animals studied one day after injection of material.

The thrombus formation was quite diffuse and appeared to involve all lobes of both lungs. In the early stage the distribution in these different lobes appeared to be of a rather uniform nature. Thrombus formation involved the pulmonary vascular bed, extending from large branches of the pulmonary arteries down to the small arteries, arterioles, and alveolar capillaries (Fig. 1). Since in the early stage the thrombotic process appeared to be quite diffuse whereas later in the stage or organization and healing it was substantially less diffuse, the indication is that some of the lesions were in some way completely resolved.

The involvement of large vessels was indicated by the presence grossly of hemorrhagic infarcts of the lung of fairly good size. Occasionally there was infarction of practically an entire lobe of lung and this was observed in several animals.

In some of the larger arteries thrombosis was evidently followed by organization which left a recanalized orifice with several eccentric lumens lined by endothelium and separated by bands of fibrous tissue showing deposits of hemosiderin and lymphocytic exudate.

A common lesion consisted of eccentric mural thrombosis which was organized, re-endothelialized, and incorporated into the wall (Fig. 2). When healed this gave an eccentric lesion of intimal fibrosis and increased elastic tissue (Fig. 3). The various stages in this process were readily observed in different animals and at 8 months after the first injection were found in a number of vessels.

There was in addition a more concentric type of lesion also characterized by intimal fibrosis and elastic reduplication (Fig. 4). There was a variation in the degree of narrowing of the vessel lumens and the narrowing was either fairly marked or minimal. The end stage of mural thrombosis organization and endothelialization, the mural plaque, was also observed (Fig. 5).

It was difficult to evaluate the change in the medial coat. Often many of the small arteries showed prominent medial layers which were richly cellular and there was a suggestion of increase in size of the nuclei. The difficulty in evaluation, however, arose from the fact that a similar change was sometimes seen in the normal control animals, although this was usually less marked and not so diffuse.

No significant change evidently occurred in the adventitial layer of the vessel. In addition, exudative inflammatory change was usually slight except for instances where organization of intimal thrombi was accompanied by some infiltrate, usually of lymphocytes (Fig. 1).

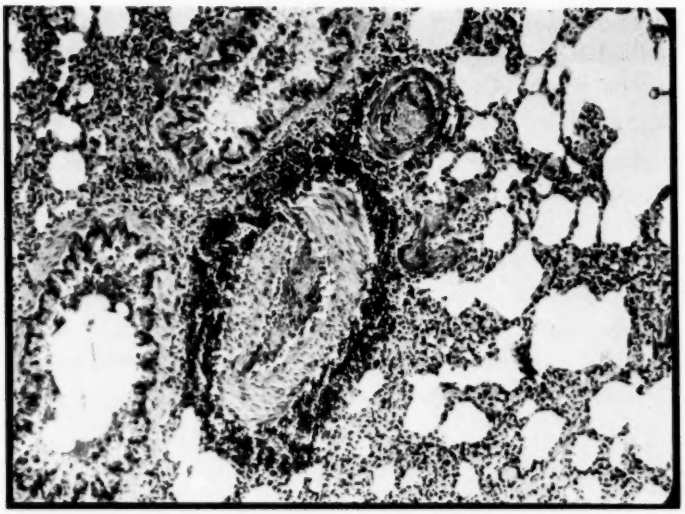

Fig. 1.—The lumens of a small artery, arteriole, and capillary are occluded by thrombi. The latter are composed of platelets, fibrin, red blood cells, and leukocytes. (Hematoxylin and eosin. ×120; reduced ¼.)

Fig. 2.—One side of the arterial wall shows an adherent fresh thrombus. On the opposite side is a mural thrombus which is partly organized and re-endothelialized. (Hematoxylin and eosin. ×180; reduced ¼.)

A sequence of lesions consisting of thrombus formation, organization, incorporation of the thrombus into the wall, fibroblastic proliferation, hyalinization, elastification, re-endothelialization of the thrombus, and eventual final incorporation completely into the intima, could be shown (Figs. 1 to 5). Exact correlations were rendered somewhat difficult by the methodology of these experiments which called for repeated injections. It is worth noting again that

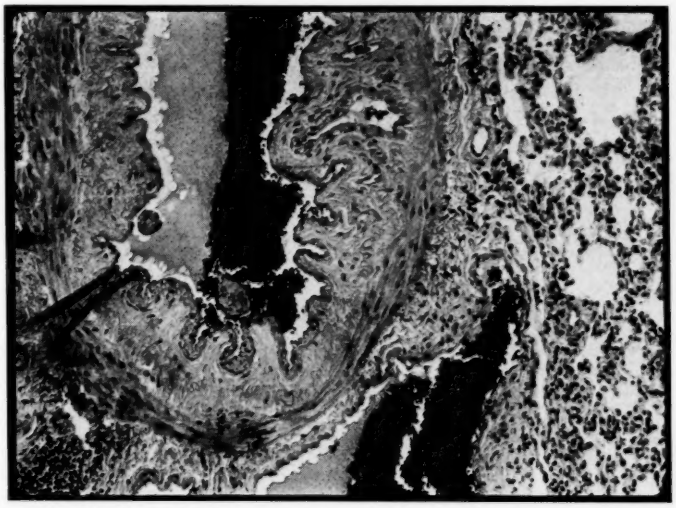

Fig. 3.—Along intima of pulmonary artery are several polypoid fibrous bands indicating healed thrombi being incorporated into intima as elastified connective tissue. (Hematoxylin and eosin. ×160; reduced ¼.)

Fig. 4.—There is advanced organization of an adherent thrombus which is largely replaced by fibroblasts. (Hematoxylin and eosin. ×140; reduced ¼.)

the formation of thrombi seemed to be substantially more widespread in the early stage of the experiment than in the later phase. In the latter there was often only a patchy or focal process of healing and organization in animals which had received from 12 to 20 injections. This again raises the question as to the resolution of some of the thrombi. It also is related to the observation that in general the vascular change observed in these rabbits was of moderately diffuse nature but generally not as diffuse or widespread as in the case in human beings with advanced pulmonary vascular disease and hypertension.

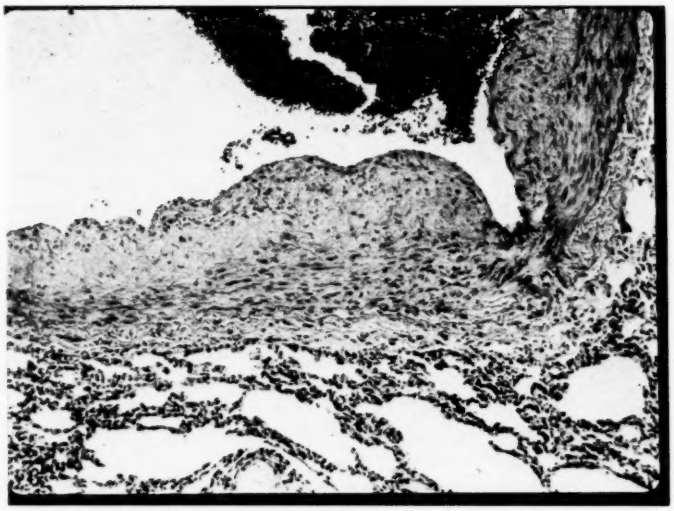

Fig. 5.—Intimal fibrous plaque of pulmonary artery. This represents the end stage of organized mural thrombi. (Hematoxylin and eosin. ×140; reduced ¼.)

DISCUSSION

The pulmonary vascular bed has interested investigators for some time because of the profound changes that occur in arteries secondary to changes in pressure and flow. Extensive research has been done in animals in an effort to understand the mechanism of these changes.[1, 2, 5, 8, 13-15, 22, 37, 41, 42] With some over-simplification, the concepts can be generally divided into three different categories: (1) changes are secondary to long-term spasm of arterioles; (2) changes in the vessels are associated with direct damage to the vessel wall associated with high pressure and flow; (3) changes are associated with endothelial damage caused by the filtering out of particulate matter, but more specifically fibrin materal that may deposit on the wall of the small vessels.

Changes Secondary to Vessel Spasm.—Those who consider vessel spasm a factor in the development of pulmonary arteriosclerosis point out that clinical conditions such as mitral stenosis in which left atrial pressure is transmitted back to the pulmonary capillary are often associated with pulmonary arteriosclerosis. Patent ductus arteriosus in which pulmonic pressure may reflect systemic pressure is often associated with the same lesion, and spasm has been felt to play an important role in the development of these changes in the pulmonary bed. Experimental studies have been of interest in this regard. Hara and Smith[13] produced obstruction of lobar arteries of dogs down to 0.5 mm. diameter with balloon catheters, dilators, pea seeds, navy beans, and glass beads without finding a significant rise in pulmonary pressure. These same investigators also found[37] that when fine catheters were introduced into lobar branches of a dog's lung and material such as starch or barium sulfate was injected, a marked rise in pulmonary arterial pressure and a marked drop in systemic blood pressure occurred with rapid death. When the pulmonay artery proximal to the

point of introduction of the catheter was clamped, these changes did not occur. From these and other findings, Whitteridge[28, 46] postulated that active pulmonary vasoconstriction occurred as the result of stimulation of receptors lying between the capillary and the higher pressure arteriole. A receptor in the precapillary site would explain the marked rise in pressure with very small emboli and the absence of pressure rise with larger emboli. Obstruction distal to the receptor would stimulate spasm of pulmonary arterioles. Occlusion of the larger vessel proximally would then abolish the reflex as is seen in the dog experiments of Smith and Hara. Teleologically then, the rise in pressure of the precapillary arteriole in both patent ductus and mitral stenosis would then lead to spasm of the arteriole which would protect the capillary from the high pressures that would otherwise exist. Presumably, long-term spasm of this sort might lead to changes such as medial hypertrophy and intimal hyperplasia associated with pulmonary arteriosclerosis.

Changes Associated With High Flow.—Damman and others[8] produced pulmonary vascular changes consisting of medial hypertrophy and intimal proliferation by anastomosing the subclavian artery and distal pulmonary artery branches. They found by serial biopsies that a gradual spread of the lesion occurred as the high pressure was transmitted further from the point of anastomosis by the loss of edema and cellular reaction in the proximal portion. Cohn and others[5] performed a similar experiment on 10 dogs of which only 4 showed changes of medial hypertrophy and intimal proliferation. These changes were not reversed when the anastomosis was eliminated. Lynn and Bahnson,[22] on the other hand, produced artificial interatrial septal defects and patent ductus arteriosus; in their animals hypertension was relieved by interruption of the ductus and no permanent organic changes were noted.

O'Neal and Thomas[27] pointed out that when patients with left-to-right shunts were compared to patients with pulmonary stenosis there was a similar incidence of pulmonary arteriosclerosis except that it is more severe in the patients with shunts. As they point out, changes occurring under such diverse conditions as very high and very low pressure certainly suggest other factors than high pressure and spasm as the only mechanism for this abnormality. They pointed out that there is a high incidence (70 per cent) of thromboembolism in patients with pulmonic stenosis and postulated that embolism was the factor responsible for the changes in media and intima.

Changes Associated With Thromboembolism.—McGuire and co-workers[24] presented 4 cases of pulmonary hypertension proved by catheterization; one had been examined at autopsy and showed no evidence of pulmonary vascular disease. They felt that the changes seen in patients with either primary or secondary hypertension are secondary to emboli and that significant reversible elevation of pressure may occur on the basis of spasm alone as was apparently true in one of their cases. Thomas and associates[41] gave repeated intravenous injections of plastic beads from 50 to 150 μ in diameter, and averaging a total weight of 1.9 grams per rabbit, and found no evidence of intimal changes, but

marked right ventricular hypertrophy. Rabbits that were receiving injections of homologous clot, however, showed evidence of intimal change. This would suggest, according to these authors, that the changes that occur in the pulmonary bed are primarily due to thrombosis and then intimal disease in association with the thrombus. Harrison,[14] however, injected two groups of rabbits with human blood clot. The first group was killed shortly after the last injection and the second group was allowed to live for 20 weeks before the animals were killed. Right ventricular hypertrophy was present in Group I but not in Group II. These findings suggested an element of spasm after injection which is reversible. Both groups showed an equal amount of vascular disease.

Wartman and associates,[43] repeating the work of Harrison[14] but using rabbit clots, described lesions similar to those reported by Harrison, widespread arterial lesions which were divided into 5 groups: (1) impaction of emboli without demonstrable alteration of the artery with spasm around the embolus; (2) impaction of embolus with a surrounding acute arteritis in both muscular and nonmuscular arteries; (3) endothelial hyperplasia in large and small arteries; (4) adhesion of an embolus to the intima of large arteries with endothelialized mural thrombi; (5) fibrotic healing of the lesions associated with an arteritis. These authors[42] have also injected emboli of filter paper fibers into rabbits and produced foreign body granulomata in either the intima or adventitia. In some instances the particles passed through vessel walls and were found in perivascular lung tissue.

The next step was to produce intravascular clotting in vivo and observe the changes, if any, in the pulmonary bed. As noted in the introduction, this was carried out independently by the present investigators and Barnard.[1, 2] Homologous thromboplastin made from rabbit brain and suspended in saline was used in both of these laboratories. As has been noted, control animals given heat deactivated material did not develop any significant lesions in the pulmonary bed. Animals given the active material, however, showed concentric thrombi, intimal hyperplasia, endothelialization, and organization of thrombi and, in some instances, suggestion of medial thickening. These lesions are similar to those described after the administration of clot suspensions. In the rabbit it can then be assumed that intermittent leakage of small amounts of substances having a thromboplastin-like activity might produce changes in the pulmonary vessels similar to those seen when pressures or flow rates are increased in other experimental animals.

Knowing that placental tissue[34] has a thromboplastic property and that abnormal implantation or trauma to the placenta may result in intravascular clotting in both rabbits and humans, one might speculate that a similar condition may exist in the human being. The possibility of this being a factor in so-called primary pulmonary hypertension might explain the high incidence of this disease reported by some authors in young postpartum females and in patients with abnormalities of globulin.[18, 25, 36]

The existence of amniotic fluid emboli to the lungs has been recognized for some time[3, 20, 21, 34, 44, 45] as has the afibrinogenemia associated with postpartum

hemorrhage.[12, 26, 32, 45] If pulmonary hypertension invariably followed afibrinogenemia, the case for intravascular clotting would be much stronger. However, the variables of pulmonary vessel spasm and fibrinolysis[22, 27, 36] may contribute to the sporadic occurrence of this syndrome. It is also possible that recurrent embolization may occur in other conditions than pregnancy either because of intercurrent disease,[11, 23, 29, 42] some clotting abnormality, or perhaps a defect in fibrinolysis which would hinder the absorption of capillary emboli.

O'Neal and Thomas[27] have pointed out the similarity between pulmonary arteriosclerosis secondary to emboli and that associated with shunts, high pressure, and high flows. Although both types of vessel insult may lead to pulmonary arteriosclerosis, they speculate that high pressures or blood flow rates may simply exaggerate the changes produced by embolization. It is difficult to determine in the individual patient who may have had severe pulmonary hypertension over a period of years whether the lesions are secondary to embolization or to hypertension per se. One of us has recently had the opportunity to follow a patient who will be reported elsewhere who developed gradual complete obstruction of the left pulmonary vein secondary to a fibrotic process. The changes of pulmonary arteriosclerosis are prominent throughout the left lung but absent in the right. These changes certainly lend credence to the idea that flow and pressure may not be the prime factors in the development of pulmonary arteriosclerosis. Whether it will be possible to differentiate late lesions on the basis of intimal changes, medial hypertrophy, and abnormalities in the elastic layers into groups that are secondary to emboli or to pressure changes depending on the predominance of the lesion remains to be seen.

SUMMARY

A group of rabbits were given repeated injections of active thromboplastin intravenously. A second group received heat inactivated thromboplastin. Studies of the pulmonary bed by the double blind technique showed that significant pulmonary vascular changes of thrombus formation, intimal hyperplasia, endothelialization, and organization of thrombi occurred in the group receiving active material, whereas these changes were not found in those animals receiving heat inactivated material.

Some of the experimental studies that have been done in an effort to produce pulmonary vascular changes in experimental animals have been reviewed. The findings in this experiment are consistent with other studies that show that pulmonary arteriosclerosis may be associated with physiologic conditions that predispose to thrombus formation in the pulmonary vessels.

The possible relationship between certain pathologic states in human beings which predispose to intravascular clotting and the development of idiopathic pulmonary arteriosclerosis has been discussed.

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URINARY EXCRETION OF 3-METHOXY-4-HYDROXYMANDELIC ACID AFTER SEVERAL STRESS SITUATIONS

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Quantitative chromatographic analysis of 3-methoxy-4-hydroxymandelic acid, a major chemically stable urinary product of both adrenaline and noradrenaline in the normal human being, was determined in six healthy experienced test pilots 1 and 3 hours after the following stress situations: forward acceleration, high altitude, heat, isolation, and a 4 hour period of psychologic tests. The urinary output of this metabolic product was significantly elevated only after the acceleration and altitude stress.

Healthy human subjects exposed to a series of real and mock forward accelerations showed a significant elevation in the urinary excretion of 3-methoxy-4-hydroxymandelic acid after both a real and mock acceleration.

The data suggest that in normal healthy subjects not exposed to a hazardous stress the urinary excretion of this metabolic product is relatively constant, but under certain specific stresses it is significantly elevated.

A MAJOR, chemically stable, product of both adrenaline and noradrenaline metabolism,[1, 8, 9] 3-methoxy-4-hydroxymandelic acid (MOMA) is excreted in the urine in much larger amounts than the parent amines.[7] Since present methods for analysis of catecholamines are difficult and require elaborate equipment, estimation of 3-methoxy-4-hydroxymandelic acid might serve as an indirect measure of catecholamine mobilization as well as an index of sympathoadrenal response to stress.

Changes in urinary levels of 3-methoxy-4-hydroxymandelic acid have been studied under conditions of increased gravitational force during which human subjects were exposed to a 6 g forward acceleration on a human centrifuge.[3] The results of this study showed that large increases in excretion of 3-methoxy-4-hydroxymandelic acid were associated with acceleration. However, an increased output of this metabolic product was noted to occur just before subjects were centrifuged. This was thought to be due to the anxiety of anticipating centrifugation. The much greater output observed after acceleration was interpreted as a direct effect of the gravitational stress superimposed on the increase caused by anticipation of this stress. This would imply that changes in levels of 3-methoxy-4-hydroxymandelic acid relate both to sympathoadrenal discharge in response to emotional factors associated with centrifugation as well as the reflex effects of the g force. These responses were separately determined by exposing subjects to a series of real and mock accelerations.

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However, it was necessary first to confirm the observation that increased excretion of 3-methoxy-4-hydroxymandelic acid is associated with acceleration. This was accomplished by using a different population of subjects. It was also of interest to measure the excretion of 3-methoxy-4-hydroxymandelic acid in response to other stress situations, particularly those associated with manned space flight. The results from such a survey might extend the usefulness of this catabolite as an indicator to other stresses anticipated in space flight.

This report describes the results from estimation of 3-methoxy-4-hydroxymandelic acid in six experienced test pilots one and three hours after the following stresses: forward acceleration, isolation, high altitude, heat, and one hour after subjects were given 4 hours of psychologic tests. Measurements of 3-methoxy-4-hydroxymandelic acid were also made on Wright Air Development Division centrifuge panel members before and after a series of real and mock accelerations.

MATERIALS AND METHODS

Stress.—Six healthy, experienced military test pilots, all potential candidates for space flight projects, were exposed to the following stress situations:

1. *Forward acceleration:* Subjects in the free-swinging cab of the Wright Air Development Division human centrifuge were exposed to two successive acceleration profiles, a forward acceleration (rate of onset 1 g per 5 seconds), with 15 second plateaus of 5 g and 8 g. During the time of centrifugation the subjects were supported by an unconventional restraint system of net and tubular frame.[4] With this restraint system subjects faced the center of rotation with the head and spine forming an angle of 90 degrees and the angle at the knees was also approximately 90 degrees. After a rest period, which averaged 5 minutes, the second profile was started, which was similar to the first, except that the head and spine formed an angle of 78 degrees with the acceleration vector and the 15 second plateaus were at 5, 8, and 12 g.

2. *High altitude:* Subjects in an altitude chamber were exposed to 42 mm. Hg barometric pressure equivalent to pressure at 65,000 feet, for one hour, dressed in a MC-1 partial pressure suit, with pressurized gloves, and a MA-2 helmet. Prior to entering the chamber, subjects were denitrogenated for 2 hours on aviator's oxygen.

3. *Heat:* In a thermal chamber, subjects were exposed to an ambient temperature of 130° F. and a relative humidity of 20 per cent for 2 hours. Air motion within the chamber was equivalent to turbulent air movement of 3 to 5 miles per hour.

4. *Isolation:* Subjects were seated comfortably in a relatively soundproof, lightproof chamber for 3 hours.

5. *Psychologic tests:* The subjects during a 4 hour period were given the following tests: Wechsler adult intelligence scale, Rorschach psychodiagnostic test, Thematic apperception test, and Draw-a-Person test.

Subjects were exposed to these stresses at a comparable time, but on different days during a one week interval.

Twenty-four hours before the week of stress-testing, and continuing until this period was completed, subjects were requested to refrain from taking drugs, ingesting coffee, tea, fruits, and foods containing vanilla. This was done in order to avoid accumulation of phenolic acids in the urine which might interfere with the detection of 3-methoxy-4-hydroxymandelic acid.

Subjects voided and discarded urine one hour before a stress. Urine was collected 1 and 3 hours after each stress. Aliquots were preserved with a crystal of thymol at minus 10° C.

The method used to expose subjects to real and mock accelerations was the following: nine healthy men, all regular members of the Wright Air Development Division centrifuge panel, were told that they would be exposed on three different days to a 12 g forward acceleration and the rate of onset was 1 g per 5 seconds. The ride at 12 g was to subjective tolerance and did not exceed a 3 minute duration. The restraint system, and the position of the subject's body (head and spine 78 degrees with the acceleration vector) were identical with the forward acceleration profile already described. The mock accelerations were identical in all respects to that of the real centrifugation except that subjects were spun at the rate of 2 r.p.m. which is equal to 0.02 g for 3 minutes.

In order to ensure that the mock rides were, in fact, unannounced, nine of the twenty-seven centrifugations were randomly selected as mock rides. The random distribution of the mock rides among the nine subjects was such that two subjects had two mock and one real acceleration; five subjects had one mock and two real centrifugations; two subjects had three real accelerations, for a total of 18 real and 9 mock accelerations. As a further precaution to ensure that the mock accelerations were unannounced, subjects were requested to refrain from discussing their centrifuge rides.

Dietary restrictions for these subjects were the same as those previously described for the test pilots.

Subjects in the real and mock acceleration experiment voided and discarded urine two hours before centrifugation. Urine samples were collected approximately one hour before and 20 minutes before acceleration. Postrun samples were collected at similar intervals after acceleration. Aliquots were preserved and stored as previously described.

To ensure an adequate volume of urine, especially after centrifugation, subjects drank 500 ml. of water immediately after voiding the one hour preacceleration samples.

Three weeks after the real and mock experiments were completed, a 2 to 3 hour urine sample was collected, comparable in time to when subjects were centrifuged. The results from the analysis of this postexperiment sample served as the control level for 3-methoxy-4-hydroxymandelic acid excretion.

Preparation of Urine Extract.—Two methods were used for preparing extracts from urine for two dimensional paper partition chromatographic analysis. In the first method an aliquot (1 to 2 ml.) from the measured volume of each sample was acidified (pH 1 to 1.5 with 3N HCl), and extracted with three successive portions of ethyl acetate (4, 2, and 2 ml.). The combined ethyl acetate extracts were evaporated to dryness at 50° C. The residue was dissolved in 0.5 ml. of absolute ethanol and evaporated in the same manner as was the ethyl acetate. The dried residue was taken up in a minimum volume of absolute ethanol (50 μl), and applied to filter paper for chromatographic analysis.

The second method was similar to the first except that the residue after evaporation of the ethyl acetate extract was dissolved in 0.6 ml. of absolute ethanol. One half milliliter of this solution was accurately transferred to a previously calibrated 1 ml. glass syringe. The loaded syringe was then placed on a specially designed semiautomatic miniature constant rate infusion pump,* which is capable of delivering 1 ml. of liquid in 50, 60, 70, 80, and 90 minutes. By this method, it was possible to deliver to the paper 0.5 ml. of extract which did not exceed a spot 7 mm. in diameter. With this method of extraction, reproducibility was ±5 per cent. With the former method reproducibility was ±9 per cent.

After the application of the extract to a sheet of Whatman No. 1 filter paper (13 by 12 inches), the sheet was stapled into a cylinder and developed for approximately 18 hours using a mixture of isopropyl alcohol, concentrated ammonium hydroxide solution, and water (40:1:9). The cylinder was then dried, rotated 90 degrees to form a new cylinder and developed for approximately 4 hours with a mixture of benzene, propionic acid, and water (12:9:1). The paper was then allowed to dry overnight in a fume hood. Visualization of

*The authors are grateful to Mr. Erich Gienapp, Bioelectronics Section, Biophysics Branch, Aerospace Medical Laboratory, Wright Air Development Division for construction of this infusion pump.

3-methoxy-4-hydroxymandelic acid was effected by spraying the developed chromatogram with a solution of diazotized *p*-nitroaniline. This was prepared immediately before spraying, by mixing in the cold, 10 ml. of 0.1 per cent solution of *p*-nitroaniline in a 2 per cent hydrochloric acid solution with an equal volume of 0.2 per cent sodium nitrite solution and 20 ml. of 10 per cent potassium carbonate solution.

The method used for detection of 3-methoxy-4-hydroxymandelic acid was essentially the same as that employed by Armstrong[2] except that the number of ethyl acetate extractions was increased from two to three, the composition of the benzene–propionic acid–water solvent system was changed from 20:14:1 to 12:9:1, and the volume of potassium carbonate solution was increased from 10 to 20 ml. These modifications resulted in better recoveries, greater separation of 3-methoxy-4-hydroxymandelic acid from other phenolic acid on the chromatogram, and a more intense purple spot.

Quantitative Analysis of Urinary 3-Methoxy-4-Hydroxymandelic Acid.—Two methods for quantitative analysis of 3-methoxy-4-hydroxymandelic acid were used. In the first method, spots of 3-methoxy-4-hydroxymandelic acid of known concentration (0.5, 1.0, 1.5, 2.0, and 2.5 mcg.) on the same chromatogram corresponding with the unknown spot from the urine extract were measured by scanning at 600 mμ with a recording densitometer, built according to the specifications of Durrum and Gilford.[9] The areas of the recorded density curves for the known concentration were measured by planimetry. A straight line was constructed by plotting the values for the areas of the standard against the corresponding known concentrations. From this "calibration" the concentration of the unknown was calculated. Only chromatograms from which a calibration curve within an accuracy of ±5 per cent was used to estimate 3-methoxy-4-hydroxymandelic acid.

In the second method, spots of known concentration (2, 4, and 6 mcg. delivered to the paper by the semiautomatic miniature constant rate infusion pump), corresponding with the unknown spot on the same chromatogram, were delineated in pencil. The colored spots were cut out and placed in elution tubes. These tubes were constructed in such a way that a microfunnel was fused at right angles to the tube.[5] Areas of the paper of the same size and sprayed with the reagent were marked out as blank determinations from the same chromatogram. The color was then eluted with 2 ml. of 2:1 mixture of absolute alcohol and 2 per cent sodium carbonate solution. After shaking the elution tube a few times during a half hour period, the tube was turned on its funnel end and inserted into a stoppered bell jar which could be evacuated. The piece of paper was then transferred to the throat of the funnel and tamped down lightly. The eluate, followed by washing with 1 ml. of the alkaline alcohol solution, was sucked through the paper pad into a graduated flask (5 ml.). The color, after being made to a volume of 4 ml., was thoroughly mixed and the extinction coefficients were determined against water at 520 mμ. For this purpose a Beckman model DU spectrophotometer was used with cells of 1 cm. light path. After subtraction of the blank values, the extinction coefficients of the reference spots were plotted against their concentrations. The amount of 3-methoxy-4-hydroxymandelic acid in the urinary extract was then calculated from this graph.

Two aliquots from each urine collection were used to compare the densitometric and spectrophotometric methods. They differed from each other by an average of 10 per cent. However, when analyzed by the spectrophotometric procedure, two aliquots, which had been chromatographed using the semiautomatic constant rate infusion pump previously described, yielded a difference of ±5 per cent. To a third aliquot was added a known amount of 3-methoxy-4-hydroxymandelic acid, 0.5 to 2.5 mcg., of which it was possible to recover on the average 92 ±6 per cent using the densitometric method of analysis. Recoveries of added 3-methoxy-4-hydroxymandelic acid, 0.5 to 6 mcg., were 95 ±4 per cent with the spectrophotometric method.

Preformed urinary creatinine was determined by the method of Van Pilsum and coworkers.[12]

TABLE I. URINARY EXCRETION OF 3-METHOXY-4-HYDROXYMANDELIC ACID 1 AND 3 HOURS AFTER STRESS (6 TEST PILOTS)

STRESS*	CONTROL*	FORWARD ACCELERATION		SIMULATED ALTITUDE 65,000 FT.		THERMAL		ISOLATION (1 HR.)	PSYCHOLOGIC TESTS (1 HR.)
		1 HR.	3 HR.	1 HR.	3 HR.	1 HR.	3 HR.		
Mean mcg. MOMA/mg. Creatinine	1.3 (110)	2.9 (250)†	1.9 (158)	2.0 (191)	1.2 (95)	1.2 (70)	1.0 (86)	1.4 (124)	1.7 (137)
±S. D.	0.32	0.54	0.61	0.72	0.24	0.31	0.47	0.40	0.69
±S. E.	0.13	0.22	0.25	0.32	0.11	0.12	0.19	0.16	0.28
Probability‡		< 0.001	< 0.1	< 0.05	< 0.5	< 0.3	< 0.2	< 0.9	< 0.3

*For description of each stress and explanation of control see the text.
†Numbers in brackets are mean excretion rates of MOMA in micrograms per hour.
‡Comparison of experimental variables with control.

RESULTS

Changes in creatinine excretion were not statistically significant when compared to the control levels. Since the creatinine content of the urine has been used as the basis for determining the amount of 3-methoxy-4-hydroxymandelic acid present,[1, 7] the micrograms of 3-methoxy-4-hydroxymandelic acid per milligram of creatinine was calculated for each subject at the time of urine collection.

Table I shows the excretion of 3-methoxy-4-hydroxymandelic acid in response to the psychophysiologic stresses previously described. The figures are the mean values of micrograms of 3-methoxy-4-hydroxymandelic acid per milligram of creatinine. In Table I, it should be noted that the control value was calculated from urine collected 3 hours after the subjects completed the isolation test. This period was arbitrarily selected because it was the only interval of sufficient duration in the 7 day stress-testing schedule during which time the pilots were free from further tests.

From Table I, it is evident that one hour after the forward acceleration stress, there was a significant increase in 3-methoxy-4-hydroxymandelic acid excretion ($p<0.001$). As shown in Fig. 1, which illustrates the mean excretion of 3-methoxy-4-hydroxymandelic as per cent change from control level, this increased 123 per cent.

TABLE II. URINARY EXCRETION OF 3-METHOXY-4-HYDROXYMANDELIC ACID IN RESPONSE TO REAL AND MOCK ACCELERATIONS

		TIME (MIN.)				
		BEFORE ACCELERATION		ACCELERATION	AFTER ACCELERATION	
	CONTROL*	−74	−11	0	+28	+89
Real Centrifugations						
Mean mcg. MOMA/mg. Creatinine	1.5 (107.5)†	1.7 (101)	2.4 (169)		3.2 (259)	1.6 (121)
±S. D.	0.34	0.95	2.0		1.3	2.6
±S. E.	0.12	0.22	0.47		0.29	0.61
Probability‡		< 0.7	< 0.1		< 0.001	< 0.8
Mock Centrifugations						
Mean mcg. MOMA/mg. Creatinine	1.5 (107.5)†	1.7 (103)	2.3 (161)		3.2 (226)	1.5 (113)
±S. D.	0.34	1.3	2.1		2.5	0.93
±S. E.	0.12	0.44	0.76		0.85	0.31
Probability‡		< 0.7	< 0.4		< 0.01	< 1.0

*Mean value—postexperiment control (see text).
†Numbers in brackets are average excretion rates of MOMA in micrograms per hour.
‡Comparison of experimental variables with controls.

As indicated in Table I, there was also an increased excretion one hour after the altitude stress ($p<0.05$). This increase was 54 per cent above the control level (Fig. 1). It is apparent (Table I) that urinary levels of 3-methoxy-4-hydroxymandelic acid showed no significant change after heat, isolation, and the psychologic test periods.

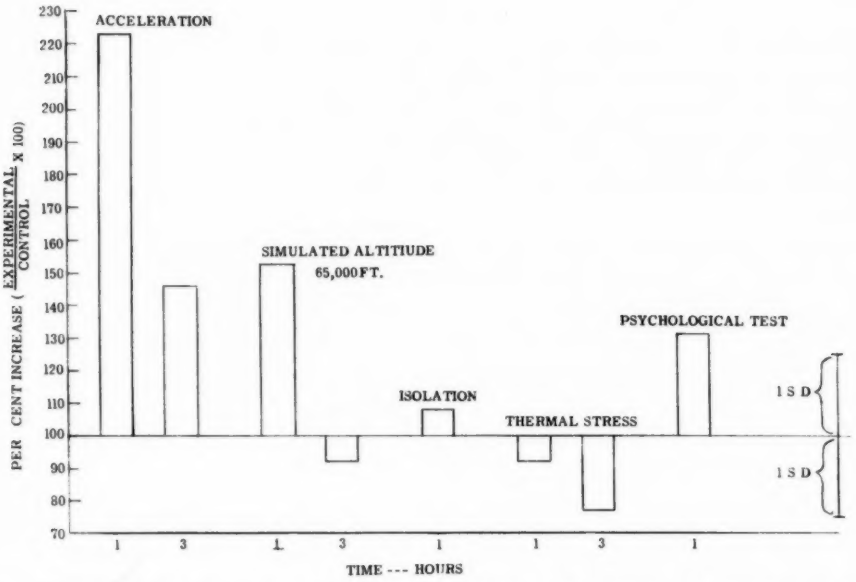

Fig. 1.—Average per cent change in urinary excretion of 3-methoxy-4-hydroxymandelic acid (micrograms per milligram creatinine) 1 and 3 hours after psychophysiologic stresses.

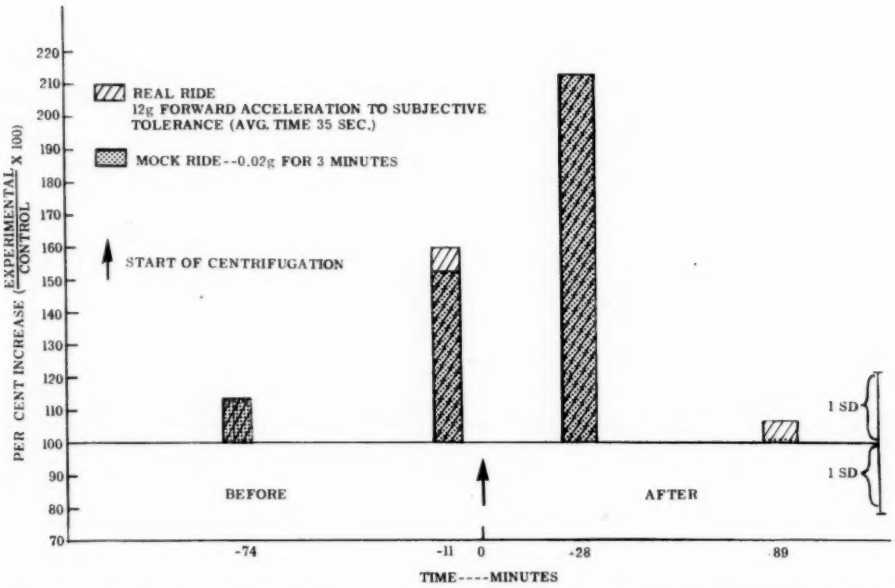

Fig. 2.—Average per cent change in urinary excretion of 3-methoxy-4-hydroxymandelic acid (micrograms per milligram creatinine) before and after acceleration.

Table II shows the mean values of the micrograms of 3-methoxy-4-hydroxymandelic acid per milligram of creatinine for each sampling period before and after a real and mock acceleration. It can be seen from Table II that there was no significant difference in this ratio between a real and a mock centrifugation. However, in the period encompassing acceleration, there was a significant increase in the excretion of 3-methoxy-4-hydroxymandelic acid compared to the control level for both a real and mock centrifugation.

Fig. 2 illustrates the average per cent change of 3-methoxy-4-hydroxymandelic acid output from the control level in response to real and mock accelerations. As shown in Fig. 2, the average per cent increase, 11 minutes before centrifugation, was 60 per cent for a real and 53 per cent for a mock acceleration. In the period ending 28 minutes after centrifugation this increase was 113 per cent above the control level for both real and mock accelerations.

DISCUSSION

The experimental evidence confirms the previous finding[3] that increased urinary levels of 3-methoxy-4-hydroxymandelic acid are associated with acceleration. The finding of an average increase in excretion of 3-methoxy-4-hydroxymandelic acid of 54 per cent ($p<0.05$) over the control level in response to the high altitude test suggests that changes in 3-methoxy-4-hydroxymandelic acid levels are also associated with this stress.

Failure to find a change in urinary output of 3-methoxy-4-hydroxymandelic acid in response to the thermal stress may have been influenced by the decreased urine volumes collected after this test.

There was no change in levels of 3-methoxy-4-hydroxymandelic acid in response to isolation (Table I). This might be expected in view of the finding[10] that the 3 hour isolation period was not stressful for this population of test pilots. No subject terminated isolation prematurely and none viewed it as a difficult stress. Most subjects slept during the isolation period.

Samples of urine collected one hour after a 4 hour psychologic test period showed that there was no significant change in excretion of 3-methoxy-4-hydroxymandelic acid. This agrees with the results obtained from the psychologic tests which indicated that these test pilots were intelligent, mature, deliberate, and confident individuals with well-integrated effective personality structures.[11]

Exposure of human subjects to a series of real and mock accelerations showed the urinary output of 3-methoxy-4-hydroxymandelic acid to be significantly elevated to the same degree after a real and after a mock centrifugation. This suggests that changes in urinary levels of 3-methoxy-4-hydroxymandelic acid reflect sympathoadrenal discharge in response to emotional factors subjects experience on being exposed to acceleration profiles of large magnitude. From this study the imposition of the g force is not responsible for the increased excretion of this metabolic product.

Since riding the human centrifuge is a hazardous stress situation, and since subjects used in this study were all experienced centrifuge riders, it might be

expected that the observed change in 3-methoxy-4-hydroxymandelic acid excretion reflects a generalized response to repetitive exposure to the originally uncertain and possible anxiety producing stress.

As previously explained, the control value for the psychophysiologic stress situations was calculated from urine samples obtained 3 hours after subjects completed the isolation test. Urine was collection at 7:00 P.M. The low excretion values found during this control period may be due to diurnal variation. Expressed as micrograms of 3-methoxy-4-hydroxymandelic acid per milligram of creatinine, the mean value for this period was found to be 1.3 ± S. D. 0.32 (Table I). The mean value for the control period in the real and mock acceleration experiment was found to be 1.5 ± S. D. 0.34 (Table II), which represents a midmorning collection, 10 to 11 A.M. Gitlow and co-workers[7] found the mean value for 15 normal healthy subjects to be 1.4 ± S. D. 0.38 for 24 hour urine collections. The close agreement of these values indicates that excretion of 3-methoxy-4-hydroxymandelic acid is relatively constant, and the control value used to evaluate the several stress situations appears to be valid.

SUMMARY

1. A densitometric and spectrophotometric method has been applied successfully to paper chromatograms, for the quantitative estimation of 3-methoxy-4-hydroxymandelic acid, a major urinary product of adrenaline and noradrenaline metabolism.

2. Determination of the output of this metabolic product in six experienced test pilots 1 and 3 hours after forward acceleration, high altitude, heat, isolation, and a 4 hour period of psychologic tests showed that the excretion was significantly elevated only after the acceleration and altitude stresses.

3. Exposure of nine human subjects to a series of real and mock forward accelerations showed the output of 3-methoxy-4-hydroxymandelic acid to be significantly elevated after both real and mock acceleration.

4. Evidence is presented which indicates that urinary excretion of 3-methoxy-4-hydroxymandelic acid is relatively constant in normal healthy subjects not undergoing a hazardous stress.

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LABORATORY METHODS

USE OF PRECURSOR PRODUCT RELATIONSHIPS IN DETERMINING SERUM ALBUMIN HALF-LIFE

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A new theoretic approach through the use of precursor product specific activity curves has been developed for the determination of a serum protein half-life. Experimental application of the theory using both carbon^{14} and nitrogen^{15} glycine provides data consistent with values previously determined by other methods.

THE rate of replacement of serum proteins, particularly albumin, has been a subject of considerable investigation in recent years. The most frequently employed technique is the in vitro radioactive iodination of albumin, followed by the study of plasma radioactivity disappearance and urinary excretion of isotope after reinjection of the protein.[1, 2] This suffers from the disadvantage that the protein studied is not identical with normal serum albumin.[3] However, recent studies in a patient with analbuminemia have shown that iodinated albumin which is prepared properly has the same disappearance rate as unlabeled albumin.[4] Also, the possibility of elution of iodine from the intact protein has been raised, and half-life differences between iodinated and in vitro "methionated" albumin have been reported as evidence for this occurrence.[5] These differences have been explained by others as a result of the use of partially denatured iodinated albumin in the experiments, since other studies gave good agreement between the disappearance rate of iodinated albumin and the methionated albumin.[6]

Estimates of albumin half-life also have been made by feeding an isotopically labeled amino acid to patients and following serum albumin specific activity.[7, 8] This approach overestimates the half-time values because of continued incorporation of isotope into albumin as has previously been pointed out.[9, 10] The theoretically least objectionable approach which has been employed is to follow the specific activity curve of albumin in a recipient given albumin which has

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been biosynthetically labeled in a donor. Some animal experiments have indicated that this method gives disappearance curves identical to those obtained by iodination of protein,[11] but others have found the iodinated protein to have a considerably shorter survival.[8] The question of denatured iodinated albumin again is raised here to explain the differences.[6] The possibility of reutilization with selective recycling, particularly with sulfur, has not been ruled out.[12]

A new approach to the problem of albumin turnover is through the use of precursor-product relationships. By measuring the precursor-product specific activity curves of free liver glycine (as reflected by hippuric acid) and of albumin glycine, it is possible to compute a corrected survival curve of endogenously labeled serum albumin which enables one to deduce the rate constant for degradation. This approach corrects for continued incorporation of isotope into the product.

It is assumed that the specific activity of urinary hippuric acid at any time is representative of synthesis of hippuric acid in the liver (and hence free liver glycine) at that time. Using both C^{14} and N^{15} glycine simultaneously, two sets of data have been obtained which allow two independent series of computations.

MATERIALS AND METHODS

Synthesis of N^{15} Glycine.—Five grams N^{15} potassium phthalimide and 5 ml. redistilled ethylchloroacetate were added to 50 ml. dimethylformamide.

The mixture was refluxed for 25 hours and allowed to cool overnight. After removal of potassium chloride by filtration, 60 ml. of water was added to the filtrate with the immediate formation of white crystals (3.84 Gm., melting point 112° C.). After the crystals were removed the dimethylformamide layer was extracted with chloroform and washed with 60 ml. 0.2N sodium hydroxide and 60 ml. water. After the chloroform was removed an additional 1.31 Gm. ethylphthalimidoacetate was obtained giving an 89 per cent yield (based on potassium phthalimide.) The ethylphthalimidoacetate was hydrolyzed to glycine hydrochloride by the method of Schoenheimer and Ratner.[13] The over-all yield of the product (identified chromatographically) was 82 per cent.

Albumin Isolation.—Albumin was initially isolated according to a modification of the method of Kendall.[14] Whole serum was chilled and 1.2 volumes of saturated $(NH_4)_2SO_4$ were added dropwise over one hour. The precipitate was allowed to form over 24 hours; the supernatant was removed after centrifugation and brought to pH 4.75 with 0.1N H_2SO_4 in half saturated $(NH_4)_2SO_4$. This precipitate was harvested by centrifugation, dissolved in water, dialyzed against water, and then against 0.05M barbital buffer at pH 8.6. The albumin was then further purified on a Porath electrophoretic column of the described dimensions[15] using "Geon"* rather than starch as the supporting medium. The column was operated at 600 volts and 25 to 32 Ma. for 20 to 24 hours using 0.05M barbital buffer at pH 8.6. The effluent was collected in 2 ml. portions and a small aliquot of each portion was analyzed by the Lowry-Folin procedure[16] for protein content. Most runs showed a small "shoulder" on the descending limb of the albumin peak (see Fig. 1). This was omitted in pooling the albumin fraction. Spot checks of albumin specimens revealed a color reaction equivalent to no more than 0.2 to 0.3 per cent hexose by the indole[17] or thymol methods,[18] a color which might result in large measure from the amino acids of the protein. Analytic paper electrophoresis of the descending limb tubes used in the pooled albumin sample (see Fig. 2) revealed pure albumin within the limits of the method, with a spread somewhat less than that seen for the albumin peak in whole serum.

*Purchased from Goodrich Rubber Co.

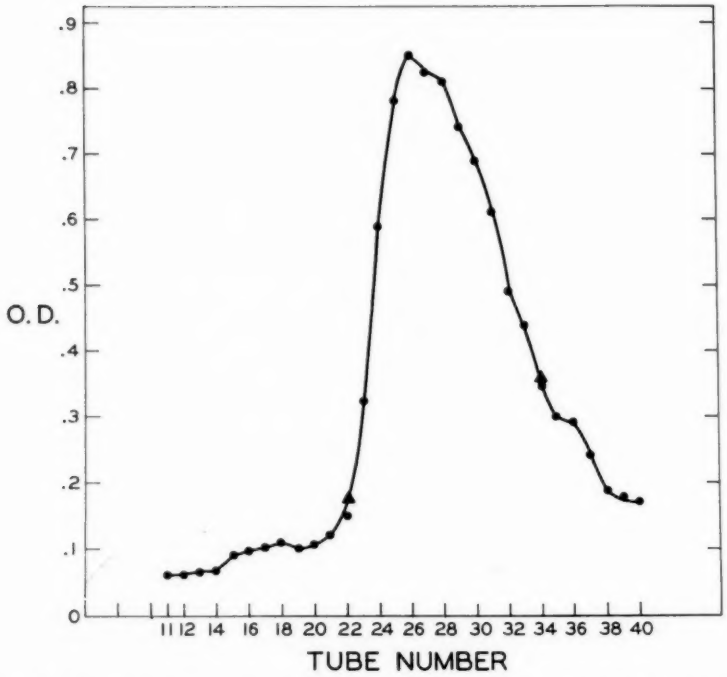

Fig. 1.—The column electrophoretic pattern of albumin purified by salting out followed by isoelectric precipitation. The contents of the tubes represented by the numbers between the triangles are those which were pooled for the purified albumin.

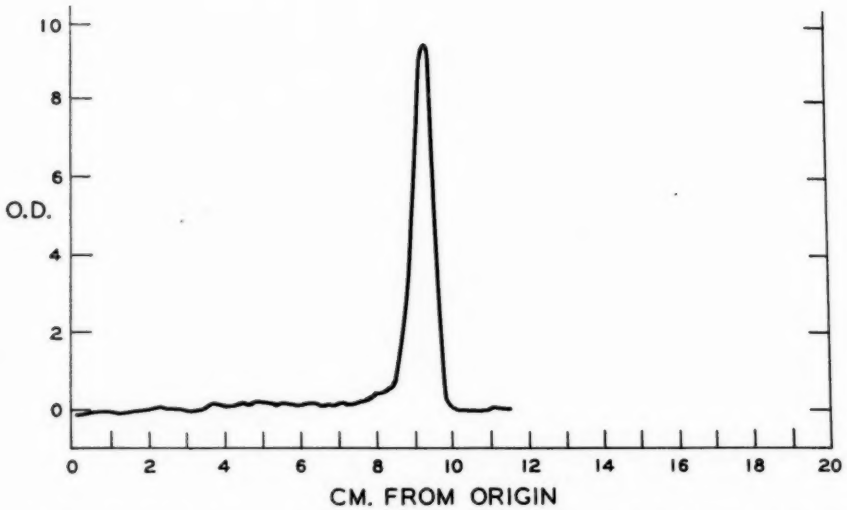

Fig. 2.—Densitometric paper electrophoresis of albumin purified by chemical fractionation followed by column electrophoresis.

Amino Acid Isolation.—Amino acids (certain data of which will be presented at a later date) were isolated by the method of Hirs and associates[19] on Dowex 1 and 50.* By lengthening the Dowex 1 column to 75 cm. it was possible to isolate phenylalanine from this column. The second column (Dowex 50) was run at 25° C. throughout. The gradient for the Dowex 50 column ran from 1N to 3.6N rather than to 4N HCl. The columns were both scaled up by a factor of two in diameter and four in volume of eluting solution. The amino acid mixture for chromatography was obtained by hydrolysis of the extensively dialyzed purified albumin with one thousand volumes of 6N HCl per weight of albumin for 18 hours, followed by evaporation in a rotary evaporator without heating above 45° C.

Amino Acid Determinations.—Effluent amino acids were quantitated by the ninhydrin method of Moore and Stein,[20] using the acetic acid eluates directly and after neutralization of an aliquot with 1N NaOH for the hydrochloric acid eluates. The specific activities of glycine could be determined only after addition of unlabeled carrier. The dilution factors computed by two methods were in good agreement. The first method consisted of two pairs of duplicate determinations by the ninhydrin method of the amino acid before and after addition of carrier. The second was based on the ninhydrin determination before addition of carrier and the known (gravimetrically determined) amount of carrier added.

Hippuric Acid Isolation.—Hippuric acid was isolated by the method of Berlin and co-workers.[21]

Isotopic Methods.—After determination of the amount of glycine isolated, 10 mg. of unlabeled carrier glycine was added. This diluted sample was divided in half and one half was used for the determination of N^{15} specific activity. The other half was further diluted by the addition of 100 mg. of unlabeled glycine. This sample was combusted to $BaCO_3$ which was counted in a liquid scintillation counter by the method of Nathan and associates.[22]

Clinical Data.—The patient was a 77-year-old white man who was found to have polycythemia very shortly before admission.

Procedure.—A total of approximately 700 mg. N^{15} was fed as glycine hydrochloride in 4 equal feedings over a period of 14 hours. One feeding was given with each of the three meals and a fourth feeding after supper. One hundred microcuries glycine-2-C^{14} was given as a single intravenous injection 3 hours after breakfast. On the day of administration of isotopic glycine the diet consisted of 2,500 calories and 15 Gm. protein in order to minimize oxidation and dilution of isotopic glycine. Blood samples (40 ml. heparinized) were drawn on days 0, 1 (2 samples, morning and evening), 2, 4, 5, 7, 9, 15, 17, 22, 36, and 65. Twenty-one samples of urinary hippuric acid were obtained over a 64 day period.

THEORY

Knowing the specific activity curves of a precursor and its product enables one to correct the product specific activity curve for continued incorporation of labeled precursor. Thus it is possible to compute values for the half-life and other kinetic parameters of the endogenously synthesized product.

It will be shown that if the curve of specific activity of a precursor and the curve which represents the specific activity of any group of simultaneously synthesized product molecules can both be represented as the sum of exponential terms, the observed curve of specific activity of product will also consist of a sum of exponential terms where the specific activity curve of the precursor is represented in the specific activity curve of the product as exponential terms which have the same decay constants as those of the precursor. The remaining terms in the observed product specific activity curve have the same decay constants as the terms in the disappearance curve of any group of simultaneously synthesized product molecules.

Proof.—The observed specific activity of glycine in circulating albumin at any time t, after administration of isotopic precursor glycine, will be the sum of the contributions made

*Obtained from the Biorad Company.

by labeled albumin glycine incorporated at all times since the administration of isotopic precursor. However, the contribution of albumin glycine, incorporated at some previous time, β (where β is any time between zero and t), will be diminished by the factor $p(t-\beta)$, less than 1, which represents the probability that a molecule of albumin synthesized at time β will still be circulating in the vascular system at time t. This probability function appears in the expression for specific activity of the product at time t and serves to correct it for mixing and degradation of albumin. A curve of p (t) would be the same shape as a curve of the disappearance of labeled albumin from the circulation and would range from an initial value of 1 to zero.

Let $G_{SA}(t)$ be the function which represents the specific activity of precursor (liver glycine as reflected by hippuric acid specific activity), G the total amount of precursor (free liver glycine), and K the rate constant for albumin synthesis (the fraction of free liver glycine incorporated into albumin per unit time). The amount of isotope which has entered the product (albumin glycine) over the interval $d\beta$ is $KG \cdot G_{SA}(\beta)d\beta$. However, at a later time t the amount of isotope incorporated during the interval $d\beta$ will be diminished by the factor $p(t-\beta)$. Thus the total amount of isotope remaining in the product at time t which had been incorporated during the interval $d\beta$ is given by $p(t-\beta)\ KG \cdot G_{SA}(\beta)d\beta$. The total amount of isotope in the product at time t (represented by A') is the sum of all the values of the expression for all intervals $d\beta$ between o and t.

$$A'(t) = \int_0^t p(t-\beta)\ KG \cdot G_{SA}(\beta)d\beta \qquad (1)$$

If A is the total amount of product (total glycine in circulating albumin) the specific activity of the product at time t is

$$A_{SA}(t) = \frac{A'(t)}{A} = \frac{\int_0^t p(t-\beta)\ KG \cdot G_{SA}(\beta)d\beta}{A} = \frac{KG}{A}\int_0^t p(t-\beta)\ G_{SA}(\beta)d\beta \qquad (2)$$

This equation is of the same form as that used by Shemin and Rittenberg[23] in their classic study of the red cell life span.

The functions $p(t-\beta)$ and $G_{SA}(\beta)$ are assumed to be the linear combination of exponential terms.

$$\text{Let } G_{SA}(\beta) = a_1e^{\alpha_1\beta} + a_2e^{\alpha_2\beta} + a_3e^{\alpha_3\beta} \ldots \text{ to n terms}$$

$$\text{Let } p(t-\beta) = x_1e^{y_1(t-\beta)} + x_2e^{y_2(t-\beta)} \ldots \text{ to q terms}$$

Equation 2 becomes

$$A_{SA}(t) = \frac{KG}{A}\int_0^t a_1x_1e^{\ y_1(t-\beta) + \alpha_1\beta} + a_1x_2e^{\ y_2(t-\beta) + \alpha_1\beta} + a_2x_1e^{\ y_1(t-\beta) + \alpha_2\beta} + a_2x_2e^{\ y_2(t-\beta) + \alpha_2\beta} \ldots \text{etc. } d\beta \qquad (3)$$

Integration of term 1 between the limits of o and t leads to:

$$\frac{a_1x_1\ (e^{\alpha_1t} - e^{y_1t})}{\alpha_1 - y_1} \qquad (4)$$

Integration of term 2 leads to:

$$\frac{a_1x_2\ (e^{\alpha_1t} - e^{y_2t})}{\alpha_1 - y_2} \qquad (5)$$

Continuing the process leads to the conclusion that the integrated expression can be expressed as the sum of n + q terms where each term contains only one different α or y in its exponent.

Data from the present experiment (Figs. 5 and 6) suggest that $G_{sA}(\beta)$ can be represented as the sum of three exponential terms and from previous data that (p)t or $p(t-\beta)$ for practical purposes is the sum of two exponential terms, one corresponding to equilibration of circulating albumin with extravascular albumin and the other corresponding to degradation.[2] More recent evidence of more than one exponential component for equilibration of circulating albumin with extravascular albumin[24] does not effect the present analysis since, as shown later, it is the exponential term corresponding to degradation which is singled out of the product specific activity curve and analyzed. The mathematical generalities presented are true for any number of exponential components in the precursor curve and curve of disappearance of simultaneously synthesized product molecules. For the sake of illustration of the theory the albumin disappearance curve will be assumed to consist of two exponential terms. Thus by adding all the terms obtained by integration it is clear that there will be only five exponential terms, each containing only one of the original decay constants (α_1, α_2, α_3, y_1, and y_2) in each exponent. The instantaneous specific activity of glycine from albumin can be represented by an expression of the form $C_1e^{\alpha_1 t} + C_2e^{\alpha_2 t} + C_3e^{\alpha_3 t} + C_4e^{y_1 t} + C_5e^{y_2 t}$. One of these terms (such as $C_5e^{y_2 t}$) corresponds to degradation of albumin. Elimination of those exponential terms contributed by the precursor (as determined here by direct measurement) gives a fairly clear choice as to which term represents the replacement of product. This term when corrected for the contribution of overlapping exponential terms allows the determination of albumin half-life.

RESULTS AND DISCUSSION

The N^{15} specific activity curves of hippuric acid, albumin, and glycine isolated from albumin are presented in Fig. 3. The corresponding C^{14} data are presented in Fig. 4. The specific activity curves of free liver glycine (as reflected by hippuric acid) and glycine isolated from albumin bear a precursor

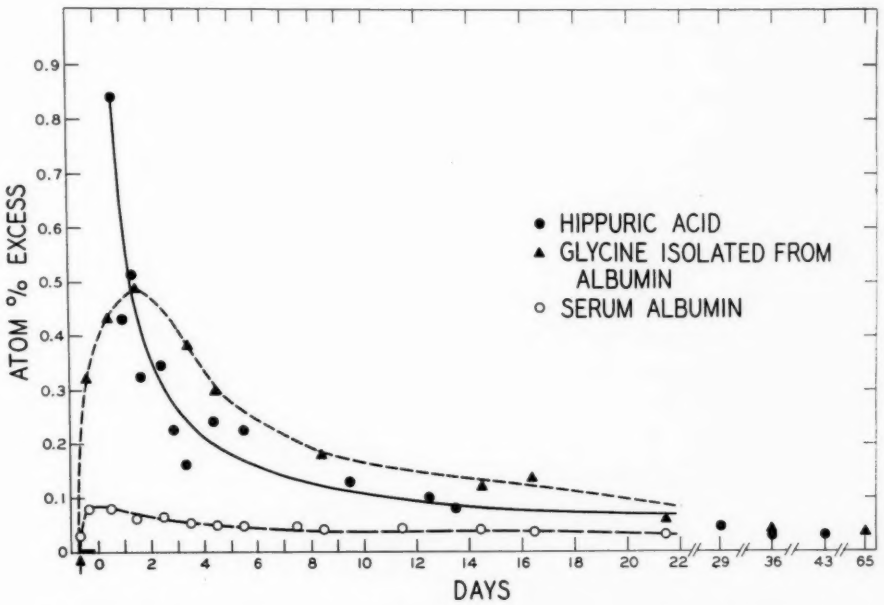

Fig. 3.—The N^{15} specific activity curves of serum albumin, urinary hippuric acid, and glycine isolated from albumin after the ingestion of N^{15} glycine.

product relationship as defined by Zilversmit[25] and Reiner[26] in the case of N^{15} and also within the limits of experimental error for C^{14}. These relationships would be expected if there were no pool (such as peptides) intermediate between free glycine and albumin, or if such a pool existed under conditions where its specific activity curve was similar to that of free liver glycine. It is clear that the precursor product relationship does not exist between the specific activity curves of free liver glycine and total albumin. This is to be expected since the specific activity of total albumin is the sum of the contributions of the specific activities of a number of amino acids which have varying time specific activity curves.

The hippuric acid specific activity curves are presented in Figs. 5 and 6 as semilogarithmic plots. They can both be represented as the sum of three exponential terms. This suggests that for rate purposes the reactions of glycine can be approximately described in three groups with rate constants (as days $^{-1}$) = 2.54, 0.111, and 0.0050 derived from the carbon specific activity curve and 0.921, 0.100, and 0.0042 derived from the nitrogen specific activity curve. These values are derived after serial corrections of each component are made for the overlapping components.

At present it is not possible to assign specific biologic meaning to the individual terms of the hippuric acid curve.

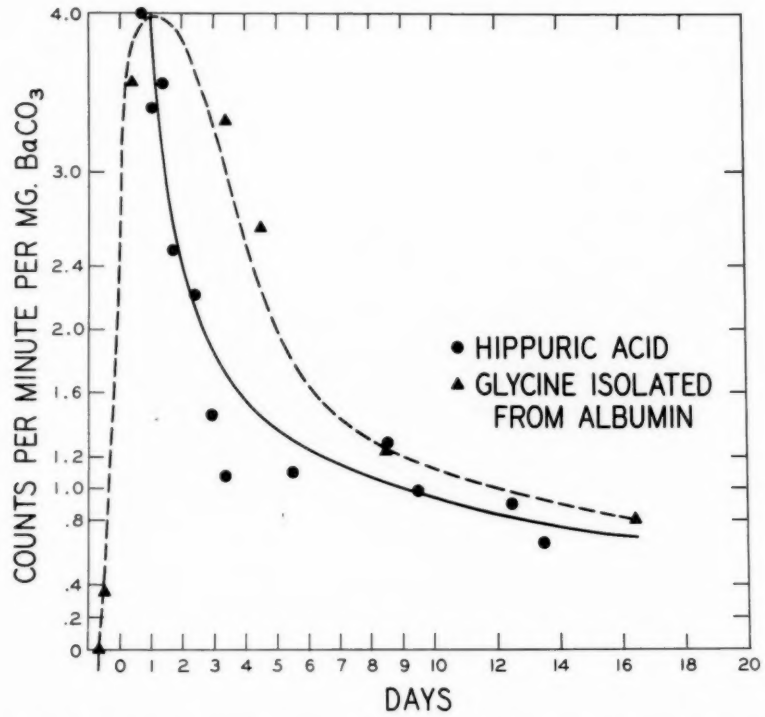

Fig. 4.—The C^{14} specific activity curves of urinary hippuric acid and glycine isolated from albumin after intravenous administration of C^{14} glycine.

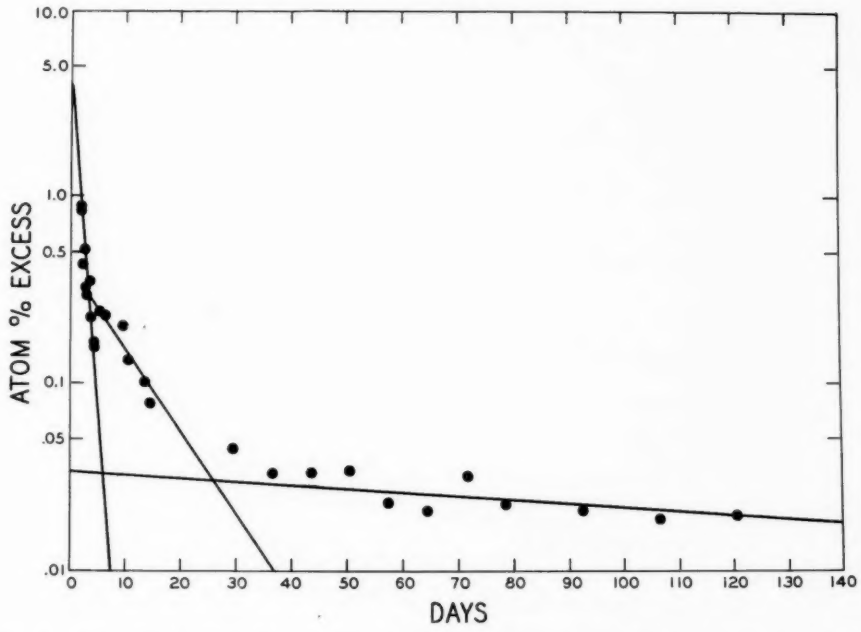

Fig. 5.—Semilogarithmic plot of hippuric acid specific activity after ingestion of N^{15} glycine.

The observed specific activity curve of glycine isolated from albumin was resolved graphically into individual exponential components by plotting the values semilogarithmically. The final linear component (designated as term L) was subtracted from the original curve. The line corresponding to term L–1 was then determined by the method of least squares. The sum of terms L and L–1 was then subtracted from the original curve and the line corresponding to term L–2 was determined by the method of least squares. The final value of term L–1 was determined by subtracting the sum of the contributions of terms L and L–2 from the original curve followed by least squares fit of a straight line to the points of term L–1. Further serial approximations did not significantly affect term L–1. By such serial corrections it is possible to approach the values which determine the slope of just one component.

In the present case the terms in the specific activity curve of glycine isolated from albumin which correspond to the precursor curve $G_{SA}(t)$ can be recognized by comparison with the precursor curve (hippuric acid). The exponential terms preceding term L–2 were obviously not those corresponding to albumin degradation. The term of $p(t)$ which is usually considered to correspond to albumin degradation is term L–1 of the semilogarithmically plotted specific activity curve of glycine. When the slope of this component is determined from the line computed by the method of least squares from the corrected points of this component a value of $K = 0.0447$ per day is obtained from the nitrogen curve corresponding to a half-life of 16 days. Theory predicts and

the N^{15} data support the theory that the slopes of the final component of the precursor (hippuric acid) and product (glycine of albumin) curves, plotted semilogarithmically, are equal.

Since the late portion of the C^{14} specific activity curve of albumin glycine was not available, it was deduced in the following way. The intersections of the various components of the semilogarithmically plotted N^{15} curve of albumin glycine were found to be at the same time points (within limits of experimental error) as those of the corresponding carbon curve. Through the time on the carbon curve corresponding to that of the intersection of the last two components of the N^{15} curve was drawn a line with the same slope as the final C^{14} hippuric acid component. By performing serial corrections as described, a value of $K = 0.0462$ is obtained corresponding to a half-life of 15 days.

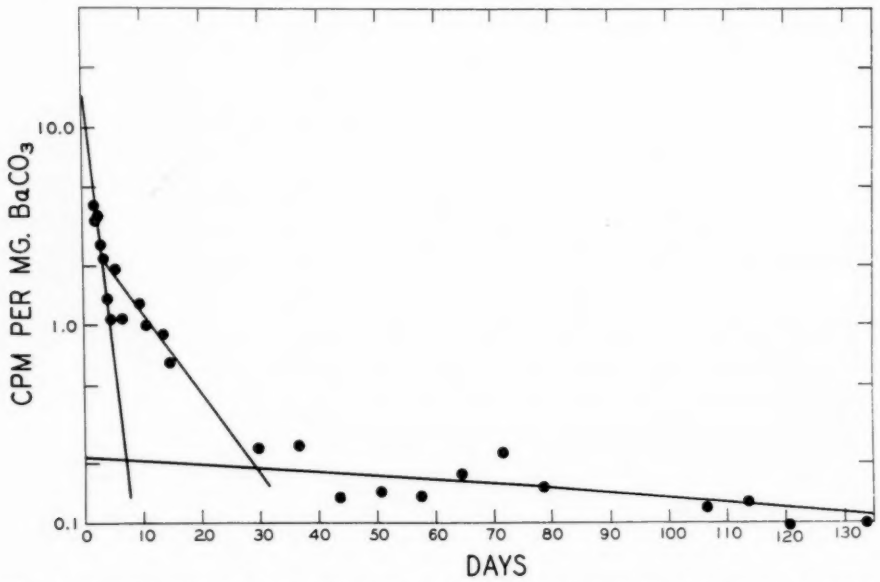

Fig. 6.—Semilogarithmic plot of hippuric acid specific activity after intravenous administration of C^{14} glycine.

Although the agreement of the N^{15} and C^{14} data is good, it should be emphasized that these values are determined by a small number of points. The data are presented primarily for the purpose of demonstrating that the application of the theory produces results which are in the range of values previously determined by other methods. Greater accuracy of the actual determination of albumin half-life could be achieved by obtaining a larger number of experimental points in the area of the curve where the degradative component is most evident. Other amino acids which have derivatives excreted in the urine which could serve for determination of precursor specific activity are arginine (using urea as the precursor), glutamine (using phenacylglutamine), and cysteine (using its conjugate with bromobenzene).

An extension of the reasoning of the theory leads to an easier experimental approach to the problem of serum protein turnover than that described previously. If two amino acids are used each containing a different isotope in a

position where the isotope would remain localized in the one amino acid, the components of the specific activity curves of the two isotopes in the protein which had common slopes would be those of the function p(t). The need for studying the precursor specific activity curve is thus obviated as is the need for isolating individual amino acids. By singling out the term of p(t) corresponding to degradation, the half-life could be estimated.

SUMMARY

1. A precursor product relationship between the specific activity curves of urinary hippuric acid and glycine of serum albumin has been demonstrated after administration of isotopic (C^{14} and N^{15}) glycine. The specific activity curves of hippuric acid can be resolved into three exponential terms.

2. A new theoretic approach through the use of precursor product specific activity curves has been developed for the determination of a serum protein half-life.

3. Experimental application of the theory using both C^{14} and N^{15} glycine provides data consistent with values previously determined by other methods.

4. A simpler experimental approach is proposed on the basis of the same theory which should allow the determination of a serum protein half-life through the use of two different isotopic amino acids without the necessity of isolating amino acids or determining the precursor specific activity.

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PROTHROMBIN MEASUREMENT BY TWO STAGE TECHNIQUE WITH OPTIMUM MOLARITY OF CALCIUM

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Two stage methods of measuring prothrombin involve the addition of tissue thromboplastin and calcium to plasma which causes generation of thrombin in the first stage; the generated thrombin is added to a fibrinogen substrate as the second stage.

The present technique eliminates the defibrination of plasma by added thrombin, and also the use of a colloid augmenter like acacia or dextran. A 37° C. working temperature is preferred to the 28° C. of previous methods. Pooled human plasma absorbed with barium is a reproducible fibrinogen solution.

The higher yield of 350 units per milliliter plasma by the present technique, despite the elimination of bovine thrombin and colloids, seems to be due to the final optimal molarity of close to 0.025M calcium.

The technique in brief: to 0.06 ml. plasma is added 0.20 ml. thromboplastin and 7.24 ml. buffered 0.025M $CaCl_2$ at 37° C. At short intervals 0.2 ml. aliquots are added to 0.2 ml. barium plasma and clotting times are determined. The unitage of prothrombin is equal to the dilution of the plasma times a factor that corresponds to the clotting time.

The prothrombin concentration by this method in normal individuals was close to 350 units per milliliter of plasma (mean, 345 ± 15).

PROTHROMBIN assay is based on measurement of the biologic clotting activity of this glycoprotein. Eventually a chemical analysis should enable a more exact assay of the prothrombin of normal human plasma. The biologic activity is measured by converting prothrombin to thrombin and reacting the thrombin with fibrinogen. Thrombin clotting activity is directly derived from the available prothrombin, and, by convention, one thrombin unit equals one prothrombin unit.

Prothrombin activity may be measured by two groups of methods: the one stage methods and the two stage methods. The one stage determination such as the method of Quick[1] measures the summation of prothrombin and accelerator globulins (Factors V, VII, and X) rather than prothrombin alone. The one stage methods consist essentially of the addition of an excess of potent tissue extract thromboplastin to plasma in the presence of an optimal concentration of calcium. The accelerated clotting time of the plasma sample is then referred to as the "prothrombin time." These prothrombin times can then be converted

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to concentration or per cent of normal by constructing a reference curve based on various dilutions of pooled normal plasmas.

The two stage prothrombin methods such as that of Ware and Seegers[2] apparently measure only prothrombin and are, therefore, more specific and accurate than the one stage methods. The two stage methods consist essentially of the addition of a dilute tissue thromboplastin to defibrinated plasma in the presence of calcium as the first stage, and then the addition of some of the generated thrombin to a fibrinogen substrate as the second stage of the test. The fibrinogen clotting time is converted into thrombin units, and the thrombin units are considered equal to the prothrombin unitage of the plasma sample.

The two stage methods have been technically difficult to perform, and there has been a lack of reproducibility from one laboratory to another.

Various modifications of the two stage methods have aimed at simplification. One recent modification proposed by Dreskin[3] used a potent thromboplastin derived from hemolyzed red blood cells, eliminated the defibrination of the plasma, and used prothrombin-free pooled human plasma as a more reproducible solution of fibrinogen. The incorporation of a colloid like acacia in the Ware and Seegers method[2] or dextran in the Owen[4] technique was unnecessary in this hemolyzed blood thromboplastin method. A 37° C. water bath was used instead of the less physiologic 28° C. water bath of the previous two stage methods. The yield in prothrombin units was 350 units per milliliter of plasma for normal people, which was slightly higher than the values obtained by Ware and Seegers,[2] and Owen.[4]

It was felt at the time of reporting the use of hemolyzed red cell thromboplastin that this material was more potent than tissue extracts. It has since been recognized that as long as the concentration of calcium is optimal (about 0.025M in the final mixture) there is little difference in potency between red cell thromboplastin and tissue extract thromboplastin. The red cell hemolyzates are somewhat less uniform than tissue extracts like commercial rabbit brain thromboplastin, and in addition the red cell hemolyzate is a partial thromboplastin dependent for full potency on the proper concentration of plasma factors like antihemophilic globulin, plasma thromboplastin component, plasma thromboplastin antecedent, etc. In contrast the acetone brain extract is a complete thromboplastin.

METHODS

The following technique is proposed for measuring prothrombin by a two stage method. The method consists essentially of mixing brain extract thromboplastin with a small amount of plasma, and adding a buffered aqueous calcium chloride solution as the first stage, and then adding an aliquot of the generating thrombin to a fibrinogen solution.

Reagents.—1. Blood (4.5 ml.) in 0.5 ml. 0.1M sodium oxalate or sodium citrate. Spin off plasma.

2. Rabbit brain thromboplastin (0.15 Gm. of the acetone dried preparation, Difco) was added to 9.0 ml. saline, and incubated with occasional stirring at 45 to 50° C. for 10 minutes. (Nine milliliters was used instead of 4.0 or 4.5 ml. to give a thin enough suspension capable of delivery through an ordinary pipette, as it has been preferred not to strain off the larger particles with cheesecloth or the like.)

3. Calcium chloride, 0.025M (buffered to pH 7.3 to 7.5 by mixing calcium chloride, anhydrous, 2.78 Gm., 0.1M barbital sodium 33 ml., 0.1N HCl 17 ml., and water sufficient to make 1,000 ml.).*

4. Fibrinogen solution (prothrombin-free plasma). Prepare as follows: to 5.0 ml. pooled fresh oxalated plasma add 1.0 ml. 30 per cent barium sulfate suspension (30.0 Gm. barium sulfate in either normal saline or water to make 100 ml.); or to 5.0 ml. pooled fresh citrated plasma add approximately 2.5 ml. solid barium sulfate by volume (about 1.6 Gm. of barium sulfate by weight). Incubate either mixture for 10 minutes at 37° C. with occasional mixing, centrifuge, and decant the prothrombin-free plasma.

Techniques.—To 0.06 ml. of oxalated or citrated plasma add 0.20 ml. thromboplastin solution and then 7.24 ml. of buffered 0.025M calcium chloride (7.5 ml. total) working in the 37° water bath. At intervals of 1, 2, 3, and 4 minutes 0.2 ml. aliquots are added to 0.2 ml. barium plasma (fibrinogen solution) at 37° C., and the clotting time is clocked. Minimal clotting times of 15 to 35 seconds are optimal. If the minimum clotting time is obtained at 1 minute, then the experiment should be repeated with intervals of ½, 1½, and 2½ minutes.

A 15 second clotting time against oxalated barium sulfate plasma at 37° C. is equivalent to approximately 1.6 units of National Institutes of Health (NIH) thrombin or prothrombin (2.0 units of NIH thrombin will clot 1.0 ml. of oxalated human plasma in 15 seconds at 28° C.; only 1.6 units are necessary to clot 1.0 ml. oxalated human plasma in 15 seconds at 37° C.); 0.06 ml. of oxalated or citrated plasma represents approximately 0.05 ml. "native" plasma. In the above experiment the 0.05 ml. "native" plasma has been diluted 150 times in the calcium chloride solution, a total of 300 times after mixing this dilution with an equal quantity of fibrinogen or barium plasma. A 15 second minimal clotting time would indicate a prothrombin unitage of 1.6 times 300 or 480 units per milliliter of plasma. Three hundred and fifty units per milliliter of plasma has been designated as 100 per cent of normal.

Conversion factors for minimal clotting times other than 15 seconds are listed in Table I. For plasma specimens low in prothrombin, the quantity of plasma should be increased to 0.12 or 0.18 ml. The final volume with calcium chloride solution may be changed to 5.0 or 10.0 ml. without changing the concentration of thromboplastin. Any final volume less than 5.0 or more than 10.0 will require proportional change in the quantity of thromboplastin.

A small clot forms at 1 to 2 minutes in the generating mixture; this may be ignored or removed from the tip of the pipette with cleansing tissue if necessary.

Serum, and likewise relatively aged plasma specimens, will require addition of fresh barium sulfate plasma to give full conversion of its prothrombin to thrombin. Thus, to 0.20 ml. of serum (0.24 ml. if oxalated or citrated) at 37° C. add 0.06 ml. fresh barium sulfate plasma, 0.20 ml. of thromboplastin, and 7.04 ml. of buffered 0.025M calcium chloride, (7.5 ml. total). At 1, 2, 3, and 4 minutes add the usual 0.2 ml. aliquots to 0.2 ml. barium plasma (fibrinogen solution). Note that the total dilution of the serum is 75 times, and the factor corresponding to the minimal clotting time will multiply this 75 to obtain the number of prothrombin units in each milliliter of serum.

RESULTS AND DISCUSSION

The prothrombin content of normal human plasma is about 350 units per milliliter by this method. These are equivalent to NIH units of prothrombin or thrombin. The NIH unit of thrombin is that amount which will clot 1 ml. of a standardized solution of fibrinogen in 15 seconds at 28° C.; 2 units of NIH thrombin will clot 1.0 ml. of oxalated human plasma in 15 seconds at 28° C. The hemolyzed red cell thromboplastin method[3] yields about 350 units per milliliter of human plasma. The classic method of Ware and Seegers,[2] and the

*Moldlike material may form in 0.1M barbital sodium solution even if refrigerated. Prepare new solution if this happens.

TABLE I. CONVERSION TABLE FOR TWO STAGE METHOD

CLOTTING TIME (MINIMAL) (SEC. AT 37° C.)	FACTOR (MULTIPLY TIMES TOTAL PLASMA OR SERUM DILUTION TO GIVE PROTHROMBIN UNITS/ML.)
11	4.00
11.5	3.60
12	3.20
12.5	2.90
13	2.55
13.5	2.25
14	1.90
14.5	1.75
15	1.60
15.5	1.50
16	1.45
16.5	1.35
17	1.30
18	1.20
19	1.10
20	1.05
21	0.95
22	0.90
23	0.80
24	0.70
25	0.65
26	0.60
27	0.58
28	0.55
29	0.53
30	0.51
31	0.50
32	0.48

Three hundred and fifty units = 100 per cent.

Fifteen second clotting time against oxalated plasma at 37° C. is equivalent to 1.6 NIH units (National Institutes of Health) of prothrombin or thrombin.

Owen[4] modification with dextran both gave 325 units per milliliter. The tosyl-arginine-methyl ester (TAMe) assay of Glueck and associates[5] yields about 54 units of prothrombin per milliliter of plasma. Because of the variation in biologic unit values, and until prothrombin can be measured by weight instead of activity, it would be best to express prothrombin concentration as a per cent of normal as well as in units.

The average prothrombin concentration by this method in 100 normal individuals was 356 units. The median was 345 units ± 15. Three hundred and fifty units was arbitrarily designated as 100 per cent. Comparison of the same patients with the Ware and Seegers[2] technique, Owen[4] method, hemolyzed red cell thromboplastin method,[3] and the present technique all gave approximately equal values in percentage of normal. The usual discrepancies were present between the one stage values of Quick's method[1] and the current values, although the correspondence was quite close when the one stage method was modified by the use of barium sulfate dilution tables instead of saline dilutions.

SUMMARY

A modified two stage method for measurement of prothrombin activity is described. The method is based on an optimum conversion of prothrombin to thrombin with acetone brain extract thromboplastin in a dilute solution that contains 0.025 mole of calcium per liter in its final concentration.

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AORTIC BYPASS PROCEDURE FOR STUDY OF GASTRIC MICROCIRCULATION

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A method is presented for quantitatively evaluating the gastric mucosal blood content in rats by densitometric measurement of the India ink content of the mucosa after the intra-aortic injection of India ink in the living animal. Since laparotomy alone may alter the circulation of the stomach an aortic bypass procedure which utilizes a polyethylene catheter through which India ink may be injected has been developed. The animals survived the bypass procedure and were kept alive in good condition for periods of 1 to 35 days. The India ink content of the gastric mucosa remained unchanged for periods of 1 to 15 days after the bypass procedure had been performed.

CONSIDERABLE interest has developed in the field of gastric microcirculation after the work of Barclay, Bentley, Barlow, and Walder[1-5] who reported the existence of a submucosal arteriovenous shunting mechanism. Most of these studies have been carried out on autopsy and surgical specimens. We have demonstrated that the gastric mucosal blood content may be evaluated quantitatively in rats by densitometric measurement of the India ink content of the mucosa after the direct intra-aortic injection of India ink into the living animal.[6] Laparotomy alone, however, may alter the circulation of the stomach presumably through splanchnic influences.[6-8] In an attempt to develop a technique which would permit the study of gastric circulation under more physiologic conditions we developed an aortic bypass procedure which utilizes a polyethylene catheter through which India ink could be injected without opening the abdomen.

MATERIALS AND METHODS

Bypass Procedure.—Sprague-Dawley, male rats (400 to 500 grams) are submitted to light ether anesthesia and xipho-pubic laparotomy is performed. The abdominal aorta is exposed between the level of the renal artery and aortic bifurcation and two cotton strings are placed behind the aorta, one at 0.2 cm. below the renal artery and the other 0.3 cm.

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above the aortic bifurcation. With an assistant holding the superior and inferior strings (to prevent hemorrhage) a small incision is made on the anterior wall of the aorta 0.2 cm. above the inferior string with the aid of a dissecting microscope.

A No. 90 polyethylene catheter (12 to 13 cm. long) is prepared by placing two small pieces of adhesive tape around it, one at 0.3 cm. from one end of the catheter (the short end) and the second at 3 cm. from the other end of the catheter (the long end). The adhesive tape serves as markers for (a) the distance from the point of insertion into the aorta to the bifurcation of the aorta (short end) and (b) the distance from the point of insertion into the aorta to the celiac axis (the long end). (The adhesive tape also serves to support the ligatures.) The catheter is filled with 10 per cent heparin solution by inserting a No. 21 needle into the long end of the catheter.

The short end of the catheter is then inserted to the point of the adhesive tape marker. The inferior string is tightened and ligated immediately distal to the marker, and the catheter is flushed with 0.5 ml. of heparin solution.

Another small incision is made in the aortic wall 0.3 cm. below the superior string, the needle and syringe are removed from the catheter, and the long end of the catheter is inserted into the aorta to the marker and ligated in position (Fig. 1). The catheter is fixed in place by knotting the pair of strings, and the muscle layer is closed with separate sutures positioning the upper part of the catheter to lie outside the muscle layer (Fig. 2).

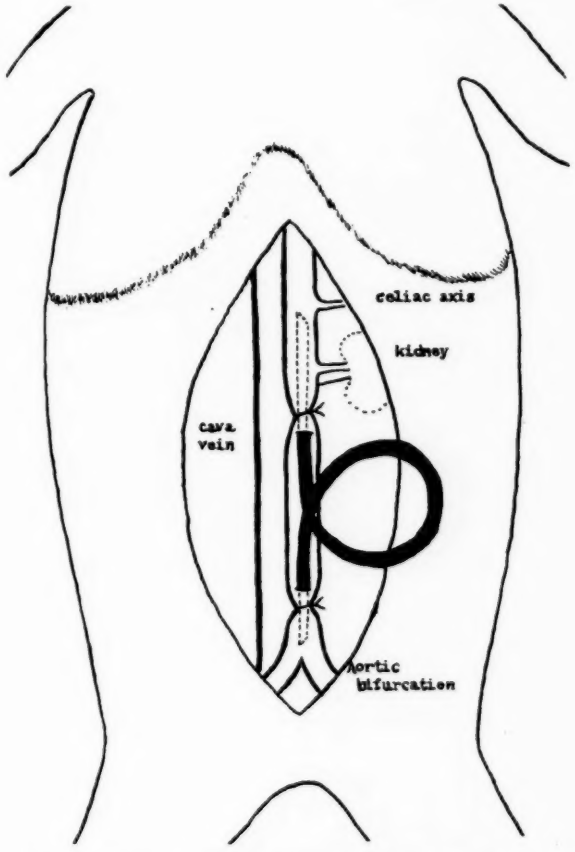

Fig. 1.—Position of polyethylene catheter in the aorta.

Fig. 2.—Appearance of the upper portion of the polyethylene catheter outside the closed muscle layer.

The skin is then closed over the catheter with skin clips, and the rat is maintained in an ordinary cage and permitted water ad libitum.

Twenty-four hours later under light ether anesthesia the skin is opened and the catheter is exposed and sectioned. A No. 21 needle is then inserted into the long end (the end pointing to the celiac axis) and the catheter is flushed with 0.5 ml. 10 per cent heparin solution. The syringe is then removed and the needle is connected to a constant pressure system that contains 25 per cent India ink solution. Approximately 4 to 5 ml. of India ink (10 per cent of the rat's body weight) is then injected at 130 to 140 mm. of mercury

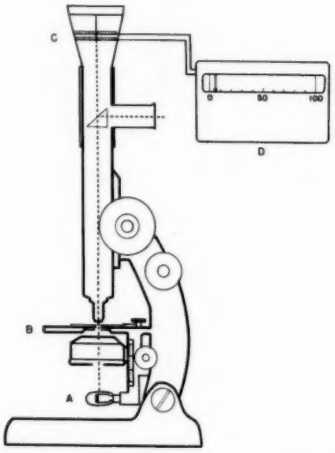

Fig. 3.—Microphotometer with photoelectric cell and galvanometric attachment. *A*, Tungsten lamp; *B*, slit or pinhole aperture; *C*, photocell; *D*, galvanometer.

pressure in 15 to 20 seconds.[6] Immediately before the India ink injection has been completed a rubber tube is placed in the rat's stomach, 5 ml. of 25 per cent formalin solution is instilled, and the tube is removed. The laparotomy muscle wound is reopened and the stomach is removed quickly with minimal trauma and placed in a beaker that contains 10 per cent formalin solution for 10 minutes, after which the stomach is opened along the lesser curvature and preserved in a clear 10 per cent formalin solution for 48 hours.

Histologic Procedure.—Sections of the fundus were obtained by two transverse parallel incisions across the mid-portion of the fundus. Paraffin blocks of the sections were made by successive passage through 70, 80, 90, and 100 per cent alcohol and xylol. The immersion time for each tissue was kept absolutely constant and uniform to obviate differences in transparency.

The microtome sections were cut at 100 μ with an approximate error of $\pm$ 10 per cent as measured by determining the absorption of light in 100 sections of noninjected specimens. The sections were then cleared through successive immersions in benzene and methyl salicylate and mounted in Canadian balsam.

Fig. 4.—Distribution of India ink in rat gastric mucosa 24 hours after the bypass procedure.

Densitometry.—The amount of India ink present in the mucosa is quantitated by determining the optical density. Relative optical densities were measured by a photoelectric cell utilizing an adaptation of a Zeiss monocular microscope with Leica photomicrographic attachments. Light was provided by a constant intensity substage white bulb. This beam passed through the substage light condenser which was set at a predetermined and reproducible distance below the stage by means of an improvised scale. The condenser's diaphragm was always maximally open or maximally constricted during a given series of measurements, depending upon the estimated optical density of the specimen. Light next passed through a 2.5 mm. diameter pinhole aperture immediately below the slide to reduce interference from reflected light and to select specific areas. The beam then transversed

the slide with the mounted specimen. After passing through the objective lens system and the Micro Ibso* attachment, the light impinged on a photoelectric cell. Responses of the cell were recorded on a sensitive pointer-type galvanometer equipped with a ratio switch for selection of low or high sensitivity (ratio 1:10) (Fig. 3).

In all experiments the condenser and diaphragm were set to give a galvanometer reading of 80 prior to interposing the glass slide and tissue section. Each reading covered an area of approximately 0.5 sq. mm. of tissue section. Fifteen readings were taken on each slide through the muscularis propia (usually poorly vascularized and containing almost no India ink). The mean of these readings was recorded and considered as a tissue blank (T.B.). Readings were then taken through the mucosa. Twenty readings were considered as minimum for each section and the average was recorded with its standard deviation (M.R.). Five sections were taken for each area studied.

The mucosal readings express the absorption of light by the tissue plus the absorption produced by the India ink present in that tissue. The difference between the reading of the tissue blank minus the mucosal reading represents the absorption of light by the India ink per se, and expresses the amount of blood in terms of relative volume.

T.B. – M.R. = amount of India ink

RESULTS

The India ink content of the gastric mucosa of the rat was measured by the densitometric procedure in 10 rats 24 hours after the bypass had been performed. The mean light absorption in these animals was 6.1 units (Table I, Fig. 4). The animals survived the bypass procedure very well and were kept alive for variable periods of time (1 to 35 days) depending on the experimental plan. The India ink content of the gastric mucosa was essentially the same when the India ink was injected into the catheter 7 to 15 days after the bypass procedure as that noted 24 hours after the bypass had been performed (Table II).

TABLE I. LIGHT ABSORPTION BY INDIA INK (DENSITOMETER UNITS) IN RAT GASTRIC MUCOSA 24 HOURS AFTER BYPASS PROCEDURE

RAT NO.	TISSUE BLANK*		MUCOSAL READING†		LIGHT ABSORPTION‡ BY INDIA INK
	MEAN	S.D.	MEAN	S.D.	
65	74.1	± 4.0	67.6	± 3.0	6.5
67	71.6	± 4.0	67.3	± 5.0	7.3
69	70.5	± 4.0	63.2	± 4.0	4.3
70	70.4	± 2.0	63.3	± 2.0	7.1
71	70.9	± 3.0	64.0	± 5.0	6.9
83	71.0	± 2.0	65.7	± 2.0	5.3
84	71.1	± 3.0	64.3	± 2.0	6.8
88	69.2	± 5.0	63.6	± 3.0	5.6
90	68.8	± 4.0	64.2	± 3.0	4.6
91	70.5	± 4.0	64.0	± 5.0	6.5
					Mean 6.1 ± 1.0

*Each tissue blank is the average of 15 different readings per rat.

†Each mucosal reading represents the mean of 150 readings through 5 different tissue sections.

‡Light absorption by India ink is obtained by subtracting mucosal reading from the tissue blank.

*Ipschaphot, photoelectric exposure meter for Micro Ibso attachment Brinkmann Instruments, Inc., Great Neck, Long Island, N. Y.

TABLE II. LIGHT ABSORPTION BY INDIA INK (DENSITOMETER UNITS) IN RAT GASTRIC MUCOSA 7 TO 15 DAYS AFTER BYPASS PROCEDURE

RAT NO.	TISSUE BLANK*		MUCOSAL READING†		LIGHT ABSORPTION‡
	MEAN	S.D.	MEAN	S.D.	
1 (7 days)	71.1	± 3.0	64.3	± 2.0	6.8
2 (12 days)	73.0	± 4.0	66.0	± 3.0	7.0
3 (15 days)	70.4	± 2.0	64.5	± 3.0	5.9

*Each tissue blank is the average of 15 different readings per rat.

†Each mucosal reading represents the mean of 150 readings through 5 different tissue sections.

‡Light absorption by India ink is obtained by subtracting mucosal reading from the tissue blank.

DISCUSSION

We have previously demonstrated that laparotomy alone may alter the gastric circulation of the stomach and its India ink content presumably through splanchnic influences. The bypass catheter technique described permits the injection of India ink into the aorta without opening the abdomen, thus avoiding changes in the microcirculation secondary to laparotomy, transection of nerve structures, handling of the viscera, etc.[6-8]

The circulation of the stomach after the bypass procedure is not significantly altered with time. The India ink content of the mucosa after injection into the catheter 24 hours after the bypass procedure is the same as that after injection of India ink 7 to 15 days later (Tables I and II).

Apparently the hemodynamics of the animals remain stable after the bypass procedure since the aortic blood pressure obtained by inserting a No. 21 needle into the catheter 24 hours after the procedure was essentially the same (90/60, mean 70 pulse 360). There was no evidence of paralysis of the hind limbs or cyanosis usually seen in distal aortic ligation, and the animals remained in good nutrition and activity.

Evidence that the histologic technique from tissue to tissue was kept uniform throughout is demonstrated by the close approximation in light absorption by the tissue blanks in the 13 animals studied (Tables I and II). The light absorption by the tissue blanks varied from 68.8 to 74.1 densitometer units with a standard deviation of ± 2.0 to 5.0. Similar reproducibility of the data by this procedure was obtained in 17 animals reported previously.[6] Different types of fixation were not investigated since the technique described yielded uniform and highly satisfactory results.

SUMMARY

1. The gastric mucosa blood content may be evaluated quantitatively in rats by densitometric measurement of the India ink content of the mucosa after direct intra-aortic injection of India ink into the living animal.

2. An aortic bypass procedure utilizing a polyethylene catheter through which India ink may be injected has been described.

3. The advantages of this technique are discussed.

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RAPID ASSAY PROCEDURES FOR TRITIUM-LABELED WATER IN BODY FLUIDS

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Two methods are presented for the accurate radioassay of tritiated water in connection with body water determinations. Both methods would be applicable to body fluids in general. Considering factors of accuracy, simplicity, and time, the method of choice is rapid vacuum sublimation. This requires a 0.5 to 2 ml. sample and is carried out in a single, individual glass unit. In cases where vacuum sublimation may not be feasible, internal standardization would give results approaching similar accuracy provided an auxillary determination is made of the water content of the original sample.

For twelve active, healthy men the mean exchangeable body water plus or minus 1 standard deviation was 51 ± 5 L. Analogously, body weight for this group was 102 ± 9 kilograms.

THE use of internal sample liquid scintillators for counting tritiated water has been described in several recent reports.[1, 2] While the technique is rapid and relatively simple in comparison with deuterium oxide techniques,[3] certain difficulties are presented by the quenching effects of plasma protein, plasma water itself, and possibly of other constituents. For the past 3 years in our laboratory, plasma, intestinal fluid, and urine samples that contain tritiated water have been radio-assayed by liquid scintillation. Both internal standardization and vacuum sublimation have been investigated as means to avoid error caused by light quenching impurities in these body fluids. Two accurate, improved techniques are described and evaluated, one for rapid vacuum sublimation where sample size is small and greatest accuracy is required, and one for simplified internal standardization where adequate sample is available.

METHODS

Scintillation Counting.—Sample counting was carried out in a liquid scintillation spectrometer.* Refrigeration was set at + 9° C., and the instrument was used according to the manufacturer's instructions, after it was established that counting response was linear over the range encountered in the samples. Discriminators were set at 10 and 50 volts, and samples were counted to a statistical error of less than ± 0.5 per cent.

Two kinds of liquid scintillant systems were used, one utilizing dioxane as the solvent, the other utilizing a toluene-ethanol mixture as the solvent. The dioxane system holds water

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*Tri-Carb liquid scintillation spectrometer, model 314C, Packard Instrument Company, Inc., La Grange, Ill.

in solution in the ratio of at least 1 ml. water per 3 ml. scintillant. It contains 7 Gm. diphenyl oxazole, 50 Gm. naphthalene, 0.050 Gm. of 1, 4-di[2-(5-phenyloxazolyl)] benzene, and 1,000 ml. of dioxane. The toluene-ethanol system holds water in solution in the ratio of 1 ml. water per 65 ml. scintillant. It contains 4 Gm. diphenyl oxazole, 0.015 Gm. 1, 4-di[2-(5-phenyloxazolyl)], 230 ml. absolute ethanol, and 770 ml. toluene. All solid reagents used were of special scintillator grade obtained commercially.* The solvents used were of analytic reagent grade.

Rapid Vacuum Sublimation.—Special tubes employing a Thunberg closure were made according to Fig. 1. Each unit is intended for samples from 0.5 to 2 ml. in amount. Normally, approximately 1.5 ml. of sample was pipetted into bulb *A*, the neck was greased (Apiezon type M) and the unit was *closed immediately.* Holding the unit by its neck, the bulb, *A*, was placed into a flask of liquid nitrogen and was swirled in order to freeze the sample as a thin shell. When boiling of the liquid nitrogen ceased the flask was removed and its side arm was attached to the line leading directly to a high vacuum pump (pressure approximately 25 μ). The Thunberg closure was rotated into the open position, evacuating the flask while the next sample was being frozen. When the next sample was frozen, the previous unit was closed, removed from the pump line, and hung over the edge of a large Dewar beaker with the receiver, *B*, just immersed in the liquid nitrogen. Usually about 30 minutes later, when all water in *A* had condensed in *B*, the unit was removed from the Dewar beaker and vacuum was released. When the contents of *B* had thawed, the receiver was removed and inverted. Exactly 1 ml. of water was transferred from the V-shaped inverted top to the counting vial, which contained 17 ml. of scintillant without added standard. The vial was closed, shaken and allowed to chill, then counted. (For *toluene* scintillant, 0.250 ml. of water was pipetted into 17 ml. of scintillant.)

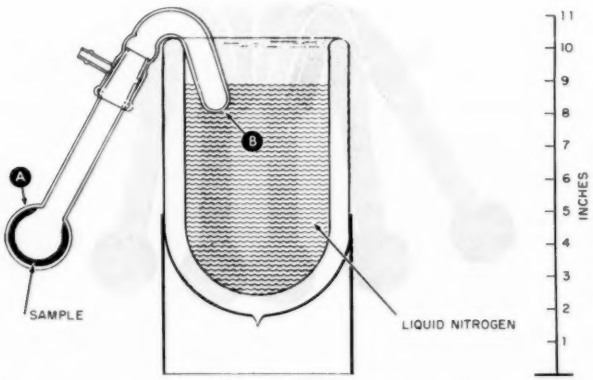

Fig. 1.—Rapid vacuum sublimation unit. *A*, Sublimation bulb. *B*, Receiver for the condensed water vapor. During sublimation, the tip of *B* is just immersed in liquid nitrogen or solid CO_2-alcohol mixture, the sample is frozen as a thin shell inside *A*, and the side arm valve is closed.

Simplified Internal Standardization Technique.—Since precipitation of plasma protein occurs on adding plasma directly to a dioxane scintillator system, counting was carried out on the supernatant fluid only. Simultaneous correction both for loss of radioactivity in the discarded precipitate and for quenching was achieved as follows: For each plasma sample, duplicate centrifuge tubes were fitted with Teflon inserts in the screw caps. In addition, duplicate tubes for a reagent blank and duplicate tubes for the internal standard were prepared at this time. An amount of liquid scintillant sufficient for all samples, blanks, and internal standards was divided into two portions. Tritiated water was added to the one portion, in the ratio of 10 μl of high specific activity tritiated water to 20 ml. of liquid scintillant, giving a counting rate of 10,000 c.p.m. per 20 ml. This is the "internally standardized"

*Supplied by Pilot Chemicals, Inc., Watertown, Mass.

portion. The other portion had nothing added to it. Seventeen milliliter quantities of scintillant were pipetted into the centrifuge tubes. Two pipettings were made of each sample, one into internally standardized scintillant, the other into nonstandardized scintillant. Tubes were capped, shaken, and centrifuged for 15 minutes at 1,000 g. The supernatant next was poured directly into counting vials, which were allowed to chill, then counted.

Calculations.—The corrected counting rate by internal standardization is derived as follows: the difference between total counting rate, sample and internal standard together, and counting rate of sample alone represents the reduced counting rate of the internal standard which results from quenching agents and loss of sample in precipitated proteins. The reciprocal of the ratio of counting rates of internal standard with and without quenching gives the necessary correction factor. This is summarized in the following calculation:

$$x_c = (\bar{x}) \cdot \frac{s}{(x + s) - (x)}$$

where $\bar{x}$ = sample net counting rate in nonstandardized scintillant; $(x + s)$ = net counting rate of sample in internally standardized scintillant; s = net counting rate of internal standard (17 ml. of standardized plus 1 ml. of nonstandardized scintillant); x_c = sample counting rate corrected for quenching and loss in precipitate.

In order to get the counting rate of the water in a plasma sample, which is required in order to determine body water, for example, one must correct for the fractional volume of plasma occupied by the plasma solids. This will be:

$$X = x_c/F$$

where F = fractional volume of water in plasma. For normal subjects this will be 0.94 per 1 ml. of plasma.* X = standardized counting rate *of water* in plasma. The calculation of body water, taken from Schloerb and co-workers[4] is simply the ratio of total radioactivity injected minus radioactivity excreted divided by the standardized counting rate of water in plasma after equilibrium. That is:

$$W = \frac{(I - U)}{X}$$

where W = exchangeable body water in liters; I = radioactivity injected in counts per minute; U = radioactivity excreted in counts per minute; X = standardized counting rate of water in plasma as given previously, and expressed in counts per minute per liter of water.

RESULTS

For the experiments shown in Fig. 2, 10 μl of high activity tritiated water was added to several bottles that contained 13 ml. of dioxane scintillant. Exactly 0.25, 0.5, 1, 2, 3, 4, or 5 ml. of water was pipetted into a counting bottle, then, in the same order, 4.75, 4.5, 4, 3, 2, 1, or 0 ml. of additional dioxane scintillant was pipetted, to make a constant volume of 18 ml. in each bottle. In a second series the same procedure was followed except that plasma was substituted for water. For the plasma data reported in Fig. 2, the internal standardization procedure described was followed except that 13 ml. of tritiated scintillant was pipetted rather than the customary 17 ml. An appropriate amount of nonlabeled scintillant was pipetted to the 0.25, 0.5, 1, 2, 3, 4, or 5 ml. of plasma in order to make an 18 ml. final volume.

In Fig. 2, the reduced counting rate as percentage of the standard counting rate is shown in relation to increasing proportions of quenching material, the latter on the abscissa. Without internally standardizing the prepared sample, its counting rate will be reduced as the proportion of quenching agent increases.

*For 100 ml. of plasma: (7.4 Gm. protein times 0.73 partial specific volume) + (0.9 Gm. NaCl equivalent times 0.28 partial specific volume) + (8.6–8.3 Gm. other constituents times 0.5 partial specific volume) = 5.9 ml. space attributable to plasma solids.

Either water by itself or plasma acts to quench the light output produced by radioactive decay of tritium. That the observed reduction in counting rate is a consequence of light quenching agents and not a consequence simply of dilution of scintillating phosphor was proved by addition of comparable amounts of solvent without scintillation phosphor and in place of water or plasma. In this case, no reduction in standard counting rate was noticed, an anticipated observation, since the scintillation phosphors are present in considerable excess.

Several features should be noticed in Fig. 2. First, as the semilogarithmic ordinate implies, small variations in concentration of quenching agent have their most marked effect on the counting rate at low concentrations. Second, the water contained in plasma makes the largest contribution to the observed quenching effect of plasma. This can be seen by comparison of the plasma and water lines of Fig. 2; whereas 2 ml. of water reduces the standard counting rate to 60 per cent of its nonquenched value, 2 ml. of plasma reduces it to 50 per cent, a not greatly different degree of quenching. Third, internal standardization gives zero-quenched counting rates for the pipetted plasma. Since plasma contains about 94 per cent water by volume, the standardized plasma radioactivity concentrations are low in comparison with ideally standardized values. The discrepancy between standardized plasma values and ideal standardization is constant at all volumes of plasma in the counting vial. Unlike the vacuum sublimations, this factor is unique to the internal standardization technique, since in the former case, the quantitative pipetting is made of the condensed pure water. Finally, as an additional consequence of the fact that plasma contains about 94 per cent water by volume, the noninternally standardized plasma line is shifted to the right and does not pass through 100 per cent at the origin.

In Table I, data are given for body water determinations based on isotopic dilution of tritiated water in the plasma. For each subject in the table, radioactivity concentrations were assayed in 3 ways: the two sets of data obtained

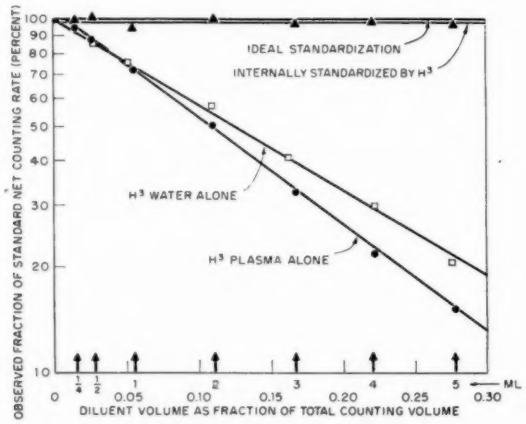

Fig. 2.—The diminution of a standard counting rate as the proportion of quenching agent increases. Counting rates are expressed as per cent of nonquenched rates. The internally standardized sample is plasma. Correction for water content has not been made for the two plasma lines. Also given on the abscissa is the volume of sample pipetted at each point.

TABLE I. PARALLEL DETERMINATIONS OF BODY WATER BY THREE INDEPENDENT TECHNIQUES IN HEALTHY ACTIVE MEN

PATIENT	BODY WEIGHT (KG.)	VACUUM SUBLIMATION PROCEDURE 1* (L.)	VACUUM SUBLIMATION PROCEDURE 2† (L.)	INTERNAL STANDARDIZATION PROCEDURE 3‡ (L.)
D	104.0	48.9§	49.7§	48.6§
		52.9‖	51.1‖	52.1‖
R	125.0	50.4	51.7	52.1
			53.3	71.5
LC	170.0	67.8	68.7	69.0
		68.1	69.6	69.7
KT	115.0	53.0	53.4	50.5
		54.0	53.9	56.9
F	112.0	45.3	49.4	48.1
		50.4	49.7	50.2
BE	122.0	53.9	54.6	54.8
		53.3	55.2	55.5
RO	83.6	43.5	45.4	45.0
		45.2	46.3	45.8
TC	79.3	49.9	50.9	51.2
		51.9	49.8	52.6
EH	83.0	50.7	53.4	58.8
		52.4	51.7	59.8
JH	78.6	59.9	53.0	57.0
		55.2	53.1	51.9
TA	98.6	48.6	48.4	46.5
		51.3	47.5	47.7
AC	58.2	38.4	37.8	36.9
		39.5	38.3	37.7
Mean	102.0	51.4	51.4	52.5
S.E. of Mean	±2.8	±1.5	±1.4	±1.9
S.D.	±9.0	±5.0		

*Toluene scintillant system was used.
†Dioxane scintillant system was used.
‡Dioxane scintillant system was used and data are corrected for a standard water content of plasma.
§Determined at 2 hours.
‖Determined at 3 hours.

by vacuum sublimation, shown in Columns 3 and 4, were carried out independently, one using conventional equipment,[3] the other using the improved apparatus described here. The set of data obtained by internal standardization, shown in Column 5, has been corrected as indicated in the description of methods by the assumed average normal value of water, 94 per cent by volume of plasma. Body water was computed according to the formula of Schloerb and associates given in the preceding section. Whereas that formula was derived for a deuterium oxide label, comparison of the data with those for a tritium label[5] shows no important difference in the equilibration time. Since in healthy human subjects labeled water has been shown to come to an equilibrium concentration in body fluids after 2 hours, a single sample taken at 2 hours can suffice for calculation of the body exchangeable water. However, because equilibrium may not be established before 6 to 8 hours in certain disease states,[6] it is desirable to determine body water at 2 and 3 hours as a check on the presumed healthy subject, as shown in Table I. For the data shown in Table I, application of the Wilcoxon test did not prove significant differences between the 2 and 3 hour determinations.

It is evident that the three independent procedures used to assay samples for body water determination give accurate results, inasmuch as each mean value is within one standard error.

DISCUSSION

The method of choice is rapid vacuum sublimation of water using the simplified glassware described here. This statement is made in consideration of accuracy of water extraction, simplicity of procedure, and economy of time. Since a completely lyophilized residue remains behind, there can be no uncertainty about the accuracy of water extraction. Furthermore, there is practically no danger of residue "popping over" into the condensate, thus enabling the procedure to be used, as it is in this laboratory, for the separation of the volatile H^3OH from other isotopically labeled solute ($Na^{22}Cl$). Second, unlike the internal standardization procedure described here, and unlike the benzene distillation procedure recently reported elsewhere,[7] it is unnecessary to carry out the auxiliary determination of water content of the original sample. This is so, because in the sublimation procedure quantitative measurement is made of the purified, condensed water, rather than the original sample, which may be variable in its water content. Third, as regards time for the complete procedures, from pipetting the original sample to the calculation of the data, vacuum sublimation has proved itself superior in practice to internal standardization. This is due to the additional time required for counting and computing twice as many samples, apart from time required for a determination of the water content of the original sample in the latter procedure.

The technique of internal standardization would be capable of the same accuracy as vacuum sublimation, if water content of the original sample is determined and taken into account. This proviso is of especial consequence in certain disease states in which the protein content of the plasma may be markedly altered.

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A SIMPLE ISOTOPE METHOD FOR RECORDING THE ACHILLES TENDON REFLEX IN MYXEDEMA

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The study of the Achilles tendon reflex is a useful diagnostic aid in the myxedematous subject. Previous methods described for obtaining permanent tracings of the Achilles tendon reflex in the normal and the myxedematous patient cannot be accomplished with equipment utilized in large clinical isotope laboratories. In lieu of previous methods a technique was designed which employs a scintillation detector and strip recorder to record permanent tracings utilizing I^{131} as a source material.

THE study of tendon reflexes is well established as a diagnostic aid in the myxedematous subject.[1, 2] Previous methods described[3, 4] for obtaining these tracings do not lend themselves to the equipment in a large clinical isotope laboratory. In lieu of these, a method was designed to obtain such recordings.

MATERIALS AND METHODS

Only normal subjects and patients with classic untreated myxedema secondary to total thyroid ablation by surgical operation and/or radioactive iodine were studied. The diagnosis

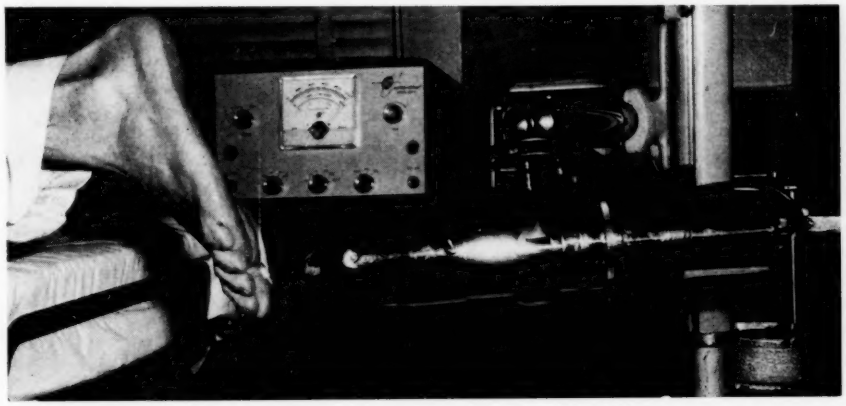

Fig. 1.—Relation of probe to source. Foot supported on sponge rubber cushion with sand bags positioned on each side of leg. Patient in prone position.

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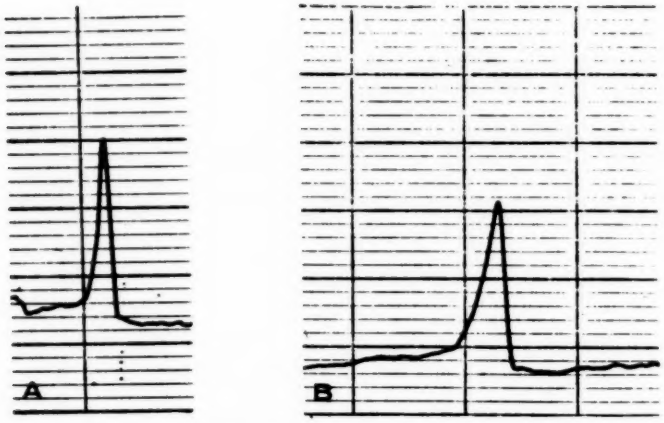

Fig. 2.—Tracing obtained using radioactive source and scintillation probe. *A*, Normal. *B*, Myxedema.

was determined by history, physical examination, 5 and 24 hour I^{131} uptake, protein-bound iodine, and red cell I^{131} uptake. A 15 μc source of I^{131}, shielded with lead to minimize radiation to the patient, was attached to the foot. A 2.5 by 2.5 cm. sodium iodide thallium activated crystal, shielded with a 1 by 5 cm. lead collimator, was connected to a ratemeter with time constant set at 0.5 second, and count range at 30 K.

Curves were inscribed on a rectilinear strip recorder run at 12 inches per minute. The probe was positioned above the source in the plane of contraction and relaxation so that on contraction of the foot the source moved into the crystal field and upon relaxation moved out of the crystal field (Fig. 1).

Recordings obtained by this method in the normal patient are characterized by a rapid upstroke, peak, and rapid return to the base line. In the myxedematous subject a delayed return to the base line was observed which resulted in a curve of larger area (Fig. 2). Curves thus obtained are similar to those described by Gilson.[4] However, they differed in that the force of the reflex hammer did not produce a paradoxical movement which could be recorded.

Investigation is being continued to correlate the rate of contraction and relaxation and total area under the curve with the clinical state. Utilizing this method it has been possible to record the Achilles reflex in the myxedematous subject before and after treatment. A delayed downstroke resulting in a curve with a wide base and area of greater magnitude than the normal has been observed in myxedematous subjects studied to date.

SUMMARY

A simple method in which radioactive I^{131} is utilized to record the Achilles reflex in the normal and the myxedematous state has been described. With the prevalence of isotope laboratories in large centers this test should be easily obtained when desired.

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